Structure and Function Relationships of Transthyretin

Ladda Leelawatwattana

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biochemistry

Prince of Songkla University

2009

Thesis Title	Structure and Function Relationships of Transthyretin
Author	Miss Ladda Leelawatwattana
Major Program	Biochemistry

Major Advisor

……..…..
(Assoc. Prof. Dr. Porntip Prapunpoj)

Co-advisor

……..…..
(Assoc. Prof. Dr. Prapaporn Utarabhand)

Examining Committee:

..……..Chairperson
(Prof. Dr. Montri Chulavatnatol)

..…….
(Assoc. Prof. Dr. Porntip Prapunpoj)

..……..
(Assoc. Prof. Dr. Prapaporn Utarabhand)

..……..
(Assoc. Prof. Dr. Nunta Churngchow)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biochemistry

...…….
(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์ ความสัมพันธ์เชิงโครงสร้างและหน้าที่ของ Transthyretin
ผู้เขียน นางสาวลัดดา ลีละวัฒน์วัฒนา
สาขาวิชา ชีวเคมี
ปีการศึกษา 2551

บทคัดย่อ

Transthyretin หรือ TTR เป็นโปรตีนที่ประกอบด้วยหน่วยย่อยที่เหมือนกันทั้งสี่หน่วย พบอยู่ในพลาสมาของสัตว์มีกระดูกสันหลังชั้นสูง มีหน้าที่หลักที่สำคัญอยู่ 2 ประการ คือ ทำหน้าที่เป็นโปรตีนขนส่งฮอร์โมนไธรอยด์และขนส่งวิตามินเอ โดยหน้าที่ประการหลังกระทำโดยอาศัยการจับกับโปรตีนขนส่งวิตามินเอที่ชื่อว่า retinol binding protein หรือ RBP นอกจากจะทำหน้าที่เป็นโปรตีนขนส่งในกระแสเลือดแล้ว ยังมีความเชื่อหรือสมมุติฐานว่า TTR มีความสำคัญหรือมีบทบาทอย่างอื่น ต่อการทำงานโดยทั่วไปของร่างกายหรือเกี่ยวข้องกับการก่อโรคบางอย่างด้วย

จากการศึกษาวิวัฒนาการของสิ่งมีชีวิตจากสัตว์มีกระดูกสันหลังชั้นต่ำขึ้นมาถึงสัตว์เลี้ยงลูกด้วยน้ำนมพบว่า TTR มีความสามารถในการเข้าจับกับ thyroxine (T_4) เพิ่มขึ้น ในขณะเดียวกันความสามารถในการจับกับ triiodothyronine (T_3) จะลดลง ซึ่งความสามารถในการเข้าจับกับฮอร์โมนไธรอยด์ที่เปลี่ยนไปนี้ พบว่ามีความสัมพันธ์กับการเปลี่ยนแปลงบริเวณปลายอะมิโน (N-terminal region) ของ TTR ทั้งในแง่ของความยาวและความมีขั้วของปลายนี้ สำหรับบริเวณปลายคาร์บอกซิล (C-terminal region) ของ TTR นั้น มีการเปลี่ยนแปลงเกิดขึ้นในระหว่างวิวัฒนาการค่อนข้างน้อยเมื่อเทียบกับบริเวณปลายอะมิโน อย่างไรก็ตาม บริเวณปลายคาร์บอกซิลของ TTR ในสัตว์มีกระดูกสันหลังชั้นต่ำ มีความเป็นขั้วน้อยกว่า บริเวณปลายคาร์บอกซิลของ TTR ในสัตว์เลี้ยงลูกด้วยนม จากการศึกษา พบความสัมพันธ์ระหว่างความสามารถของ TTR ในการเข้าจับกับฮอร์โมนไธรอยด์ที่เปลี่ยนแปลงไปกับการเปลี่ยนแปลงที่บริเวณปลายอะมิโน ประกอบกับการที่บริเวณปลายอะมิโนและปลายคาร์บอกซิลของ TTR ตั้งอยู่ ณ บริเวณทางเข้าสู่ใจกลางภายในโมเลกุล TTR ซึ่งมีตำแหน่งสำหรับให้ฮอร์โมนไธรอยด์จับอยู่ นำมาสู่สมมุติฐานที่ว่า การเปลี่ยนแปลงที่เกิดขึ้นกับโครงสร้างบริเวณปลายอะมิโนหรือปลายคาร์บอกซิลของ TTR นั้น น่าจะส่งผลต่อการที่ฮอร์โมนไธรอยด์ รวมทั้ง ligand ตัวอื่น เช่น RBP เข้าจับ ณ ตำแหน่งจับภายในโมเลกุลของ TTR

ในการศึกษาถึงอิทธิพลของโครงสร้างบริเวณปลายอะมิโนและปลายคาร์บอกซิล ที่มีต่อการทำหน้าที่ของ TTR ในวิทยานิพนธ์นี้ได้ทำการสังเคราะห์ recombinant TTR และ chimera ของ TTR ขึ้นมา โดยอาศัยระบบการการสังเคราะห์โปรตีนของยีสต์สายพันธ์ *Pichia pastoris*

ในการผลิต และได้ใช้ recombinant TTR ที่สังเคราะห์นี้ จำนวน 7 ชนิด คือ recombinant wild type TTR ในคน (recombinant huTTR), TTR ในคน ที่ถูกเปลี่ยงแปลงบริเวณปลายอะมิโนให้เหมือนกับบริเวณปลายอะมิโนของ TTR ในจระเข้ (croc/huTTR), TTR ในจระเข้ ที่ถูกเปลี่ยงแปลงบริเวณปลายอะมิโนให้เหมือนกับบริเวณปลายอะมิโนของ TTR ในคน (hu/crocTTR), TTR ในจระเข้ ที่ถูกเปลี่ยงแปลงบริเวณคาร์บอกซิลให้เหมือนกับบริเวณปลายคาร์บอกซิลของ TTR ในหมู (pigC/crocTTR) และ TTR ในจระเข้ที่ไม่มีบริเวณปลายอะมิโน (truncated crocTTR) ซึ่งผลิตจากโคลน (clone) ที่สร้างขึ้นมาใหม่ และ recombinant wild type TTR ในจระเข้ (crocTTR) และ TTR ในจระเข้ที่ถูกเปลี่ยงแปลงบริเวณปลายอะมิโนให้เหมือนกับบริเวณปลายอะมิโนของ TTR ในกบ *Xenopus laevis* (xeno/crocTTR) ซึ่งผลิตขึ้นจากโคลนที่สร้างขึ้นและมีอยู่แล้วในห้องปฏิบัติการ เป็นเครื่องมือในการศึกษา จากผลการทดลองพบว่าสามารถสร้าง cDNAs สำหรับ TTR แต่ละชนิดขึ้นได้ โดยอาศัยเวคเตอร์ pPIC3.5 ในการนำ cDNA สำหรับ huTTR เข้าสู่เซลล์ยีสต์ และเวคเตอร์ pPIC9 ในการนำ cDNAs สำหรับ TTR ชนิดอื่นๆ เข้าสู่เซลล์ยีสต์ พบว่า ยีสต์สามารถสร้าง TTR ชนิดต่างๆ ได้ รวมทั้งมีการหลั่ง TTR ที่สังเคราะห์ขึ้นมาออกสู่นอกเซลล์ยีสต์ได้ และจากการศึกษาคุณสมบัติทางเคมีกายภาพของ recombinant TTR ชนิดต่างๆ พบว่า TTR ดังกล่าวมีคุณสมบัติต่างๆ เช่นเดียวกับ TTR ที่พบในธรรมชาติ ไม่ว่าจะเป็น น้ำหนักโมเลกุลหน่วยย่อย, น้ำหนักโมเลกุลของ TTR tetramer รวมทั้งความสามารถในการจับอย่างจำเพาะกับแอนติบอดี

ผลการศึกษาถึงความสามารถในการที่ฮอร์โมนไธรอยด์เข้าจับกับ TTR แต่ละชนิด แสดงให้เห็นว่า huTTR สามารถจับกับ T_4 ได้ดีกว่า T_3 โดยมีค่า K_d ต่อการจับ T_3 เท่ากับ 53.26±3.97 nM และ K_d ต่อการจับ T_4 เท่ากับ 19.73±0.13 nM และมีอัตราส่วนค่า K_d T_3/Kd T_4 เป็น 2.70 ในขณะที่ hu/crocTTR สามารถจับกับ T_4 ได้ดีกว่า huTTR และ crocTTR โดยมีค่า K_d ต่อการจับ T_4 เท่ากับ 22.75±1.89 nM แต่ความสามารถในการจับกับ T_3 ใกล้เคียงกับ crocTTR ที่เคยมีปรากฏในรายงาน โดยมีค่า K_d ต่อการจับ T_3 เท่ากับ 5.40±0.25 nM และมีอัตราส่วนค่า K_d T_3/K_d T_4 เท่ากับ 0.24 สำหรับ truncated crocTTR พบว่าสามารถจับกับT_4 ได้ดีพอกันกับการจับ T_3 โดยมีค่า K_d ต่อการจับ T_3 เท่ากับ 57.78±5.65 nM และ K_d ต่อการจับ T_4 เท่ากับ 59.72±3.38 nM โดยมีอัตราส่วนของค่า K_d T_3/K_d T_4 เท่ากับ 0.98 ซึ่งจากผลการศึกษาในครั้งนี้ สามารถยืนยันสมมุติฐานที่ว่าบริเวณปลายอะมิโนของ TTR ที่มีโครงสร้างไม่แน่นอน มีอิทธิพลต่อความสามารถในการที่ TTR เข้าจับกับฮอร์โมนไธรอยด์

สำหรับการศึกษาถึงความสามารถในการที่ TTR แต่ละชนิดเข้าจับกับ RBP นั้น ผลการศึกษาพบว่า crocTTR และ chimera ชนิดต่างๆ ที่มีต้นกำเนิดจาก crocTTR สามารถจับกับ RBP ได้ดีกว่า TTR ที่สกัดจากพลาสมาของคน และ croc/huTTR โดยที่ TTR ที่สกัดจากพลาสมาของคน และ croc/huTTR มีค่า K_d ต่อการจับ RBP สูง กล่าวคือ TTR ที่สกัดจากพลาสมาของคน มีค่า K_d ต่อการจับ RBP เท่ากับ 17.08±3.25μM และ croc/huTTR มีค่า K_d

ต่อการจับ RBP เท่ากับ 22.52±5.84 μM สำหรับ crocTTR และ chimeras ที่มีต้นกำเนิดจาก crocTTR ยกเว้น hu/crocTTR จะมีค่า K_d ต่อการจับ RBP อยู่ในประมาณช่วงเดียวกัน คือ crocTTR มีค่า K_d ต่อการจับ RBP เท่ากับ 2.54±0.23 μM pigC/crocTTR มีค่า K_d ต่อการจับ RBP เท่ากับ 2.34±0.45 μM xeno/crocTTR มีค่า K_d ต่อการจับ RBP เท่ากับ 1.66±0.27 μM และ truncated crocTTR มีค่า K_d ต่อการจับ RBP เท่ากับ 1.19±0.19 μM ในขณะที่ค่า K_d ต่อการจับ RBP ของ hu/crocTTR สูงกว่า crocTTR และ chimeras ที่มีต้นกำเนิดจาก crocTTR กล่าวคือ มีค่า K_d ต่อการจับ RBP เท่ากับ 11.31±0.07 μM ซึ่งผลการทดลองเหล่านี้ ชี้ให้เห็นว่า บริเวณปลายอะมิโนของ TTR มีบทบาทต่อความสามารถในการที่ TTR เข้าจับกับ RBP โดยที่ปลายอะมิโนที่สั้นและมีความชอบน้ำ (shorter and more hydrophilic) จะมีอิทธิพลมากกว่าปลายที่ยาวและไม่ชอบน้ำ (longer and more hydrophobic) ส่วนปลายคาร์บอกซิล พบว่าไม่มีอิทธิพลต่อการเข้าจับกันระหว่างโปรตีน 2 ชนิดนี้

ผลการศึกษาถึงอิทธิพลของบริเวณปลายอะมิโนและปลายคาร์บอกซิลที่มีต่อคุณสมบัติความเป็นโปรตีเอสของ TTR แสดงให้เห็นว่าความสามารถในการเร่งปฏิกิริยาการสลาย ทั้ง FITC-casein และ apoA-I ของ TTR เปลี่ยนแปลงไปจากเดิม เมื่อมีการเปลี่ยนแปลงบริเวณปลายอะมิโนหรือปลายคาร์บอกซิลเกิดขึ้น และเมื่อเปรียบเทียบความเร็วต้นของการเร่งปฏิกิริยา (V_0) การสลายที่ใช้สับสเตรทจำเพาะ คือ apoA-I พบว่า croc/huTTR มีค่า V_0 ต่ำกว่า recombinant huTTR โดยที่ recombinant huTTR มีค่า V_0 เท่ากับ 17.07±0.64 nM/min และ croc/huTTR มีค่า V_0 เท่ากับ 5.79±0.50 nM/min สำหรับ crocTTR มีค่า V_0 เท่ากับ 12.77±2.05 nM/min ซึ่งสูงกว่า hu/crocTTR ซึ่งมีค่า V_0 เท่ากับ 9.91±1.11 nM/min ถึง 0.78 เท่า ส่วน xeno/crocTTR และ pigC/crocTTR มีค่า V_0 สูงเป็น 2.39 และ 3.66 เท่าของค่า V_0 ใน crocTTR ตามลำดับ โดย xeno/crocTTR มีค่า V_0 เท่ากับ 30.48±3.94 nM/min และ pigC/crocTTR มีค่า V_0 เท่ากับ 46.72±1.75 nM/min นอกจากนี้ พบว่า truncated crocTTR มีค่า V_0 ต่ำที่สุดและเป็น 0.3 เท่าของค่า V_0 ใน crocTTR โดย truncated crocTTR มีค่า V_0 เท่ากับ 3.86±1.62 nM/min ซึ่งจากผลการทดลองที่ได้นี้ แสดงให้เห็นถึงอิทธิพลของบริเวณปลายอะมิโนและคาร์บอกซิลที่มีต่อความเร็วต้นของการเร่งปฏิกิริยาการสลายของ TTR โดยที่ความยาวทั้งของปลายอะมิโนและปลายคาร์บอกซิล รวมทั้งคุณสมบัติชอบน้ำหรือไม่ชอบน้ำ (hydropathy) ส่งผลให้เกิดการเปลี่ยนแปลงโครงรูปโดยรวมของ TTR ซึ่งการเปลี่ยนแปลงโครงรูปนี้ อาจส่งผลต่อความสามารถในการเร่งปฏิกิริยาทั้งในทิศทางที่เพิ่มหรือลดความสามารถ นอกจากนี้จากผลการทดลองที่พบว่า apoA-I ที่ถูกย่อยแล้วจะมีความไวต่อการถูกย่อยสลายเพิ่มขึ้นนำไปสู่สมมุติฐานการทำหน้าที่กำจัด amyloid apoA-I ของ TTR อีกด้วย

Thesis Title	Structure and Function Relationships of Transthyretin
Author	Miss Ladda Leelawatwattana
Major Program	Biochemistry
Academic Year	2008

ABSTRACT

Transthyretin or TTR is a homotetrameric protein that found in plasma of higher vertebrates. It has two main well known functions, one is a transporter of thyroid hormones (THs) and another is a retinol carrier through the binding with the retinol binding protein (RBP). Aside functions as a protein carrier in blood circulation, TTR has been believed to have other unidentified functions involving in both physiology and pathology. During evolution of mammalians from its ancestors, the binding affinity of TTR for thyroxine (T_4) increased whereas that for triiodothyronine (T_3) decreased. This change in the binding affinity is related to the change in length and hydropathy at the N-terminal region of TTR in vertebrate during evolution. The primary structure of C-terminal region is much less changed during evolution of vertebrates. However, this region is more hydrophobic in lower vertebrate than in mammalians. Since the N- and C-terminal regions locate at the entrance to the central channel of TTR where the binding sites for THs exist, the question arises whether changes in structure of the N-terminal or C-terminal segment of TTR subunit have affect to the accession of THs and other ligand to the TTR binding site as well as other functions of TTR.

In order to elucidate the influence of the primary structure of the N- and C-terminal regions on the biological functions of TTR, the recombinant TTRs and their chimeras were synthesized using the heterologous protein expression of *P. pastoris*. Seven recombinant TTRs were produced in this thesis. Among these TTRs, five were from the new constructed clones including the recombinant wild type human TTR (recombinant huTTR), the recombinant huTTR that the N-terminal region was changed to that of *C. porosus* TTR (croc/huTTR), the recombinant *C. porosus* TTR that the N-terminus was changed to that of human TTR (hu/crocTTR), the recombinant *C. porosus* TTR that the C-terminus was replaced by the C-terminal

region of pig TTR (pigC/crocTTR) and the *C. porosus* TTR that lacked the N-terminus (truncated crocTTR). The other two recombinant clones were available in laboratory. These were the recombinant wild type *C. porosus* TTR (crocTTR) and recombinant *C. porosus* TTR that the N-terminal region was replaced by the N-terminus of *Xenopus laevis* TTR (xeno/crocTTR). The cDNAs coding for all recombinant TTRs were successfully constructed in the pPIC3.5 (for recombinant huTTR) and in pPIC9 (for the other TTRs). The cDNAs were expressed and TTRs were synthesized in cells of *P. pastoris*. The TTRs were extracellularly secreted into culture medium. The subunit mass, molecular weight of TTR tetramer and reactivity to the specific antibodies against TTR were determined. It showed that all recombinant TTRs produced by *Pichia* had all physiochemical properties similar to those vertebrate TTRs found in nature.

Analysis of the binding affinity to THs of the recombinant huTTR showed a dissociation constant (K_d) for T_3 of 53.26±3.97 nM and for T_4 of 19.73±0.13 nM, led to the K_d T_3/K_d T_4 ratio of 2.70. The affinity for T_4 of recombinant chimeric hu/crocTTR (K_d was 22.75±1.89 nM) was higher than those of wild type human TTR and *C. porosus* TTR. However, the affinity for T_3 (K_d was 5.40±0.25 nM) was similar to that previous reported for *C. porosus* TTR led to the K_d T_3/K_d T_4 ratio of 0.24. A similar affinity for both T_3 (K_d was 57.78±5.65 nM) and T_4 (K_d was 59.72±3.38 nM) with the K_d T_3/K_d T_4 ratio to 0.97 was observed in truncated crocTTR. These results strongly revealed that the unstructured N-terminus of TTR critically influences the specificity and binding affinity to THs of TTR.

The binding affinity to human RBP of the recombinant TTRs revealed that the recombinant crocTTR and its chimeras had higher affinity for RBP than the native human TTR purified from plasma and the recombinant chimeric croc/huTTR. The native human TTR and croc/huTTR possessed high K_d value for RBP, and these were 17.08±3.25 µM and 22.52±5.84 µM, respectively. The K_d for RBP of crocTTR and its chimeras except hu/crocTTR were in the same range (the K_d values were 2.54±0.23 µM, 2.34±0.45 µM, 1.66±0.27 µM, 1.19±0.19 µM for crocTTR, pigC/crocTTR, xeno/crocTTR and truncated crocTTR, respectively), whereas the hu/crocTTR showed high K_d value of 11.31±0.07 µM. These results suggested that the N- but not the C-terminal region had influence on the binding of TTR to RBP, and

the shorter hydrophilic N-terminus seems to bring more effect than the longer and more hydrophobic N-terminus.

The influence of N- and C-terminal regions on the proteolysis property of TTR was studied and the results showed that the chimeric TTRs those N- or C-terminal region was altered had different activity in comparing to the wild type TTR from either human and *C. porosus*. In the presence of the specific substrate, apoA-I, the croc/huTTR showed to has lower activity (V_0 was 5.79±0.50 nM/min) in comparing to that of the recombinant wild type human TTR (V_0 was 17.07±0.64 nM/min). The catalytic activity of crocTTR was 0.78 folds greater than that of hu/crocTTR (V_0 were 12.77±2.05 nM/min and 9.91±1.11 nM/min for crocTTR and hu/crocTTR, respectively). The xeno/crocTTR and pigC/crocTTR showed high catalytic activity with V_0 of 30.48±3.94 nM/min and 46.72±1.75 nM/min, respectively. These were 2.39 folds (xeno/crocTTR) and 3.66 folds (pigC/crocTTR) greater than the wild type crocTTR. In addition, the truncated crocTTR showed the lowest actiivty (V_0 was 3.86±1.62 nM/min) which was 0.3 folds lower than that of the wild type crocTTR. The results obtained could demonstrate to the influence of N- and C-terminal regions on the catalytic activity of TTR. Length and hydropathy of the N- or C-terminus may affect on conformation of the TTR subunit and/or molecule, which led to changes in the activity either in promote and inhibit manner. In addition, based on the finding that the cleaved apoA-I was more susceptible to further degradation, clearance of amyloid apoA-I from blood circulation was postulated as a function of TTR.

ACKNOWLEDGEMENTS

I would like to acknowledge several encouraging persons who gave me the possibility to complete this thesis.

First, I am very grateful to my supervisor, Assoc. Prof. Dr. Porntip Prapunpoj for her continuous support in the Ph.D. program. Dr. Porntip was always there to listen to my problems and to give advice since I was a master degree student. She is responsible for involving me in this research in every steps. She showed me different ways to solve research problem by using scientific idea, not only a good model as teacher she is but also a good scientist I have learned from her. Her kindness and understanding will be memorized forever. My sincere appreciation is also expressed to my co-advisor, Associate professor Dr. Prapaporn Utarabhand for her kindness, comment on my research and critical reading of this manuscript.

I would like to express my appreciation to Professor Emeritus Dr. Montri Chulavatnatol and Assoc. Prof. Dr. Nunta Churngchow, the thesis examining committees for their helpful suggestions and efforts thoughout the thesis examination.

Special thanks are also due to all the teaching staff that gave me a chance to learn the world of Biochemistry. Especially, I would like to thank Dr. Rapeeporn Sotthibandhu and Assoc. Prof. Dr. Nongporn Towattana for their kindness and encouragements.

I also would like to thank for the scholarships supported from the University Academic Excellence Strengthening Program in Biochemistry and the Graduate School of Prince of Songkla University.

I also thank all technicians and staff that gave me good helps. And, let me say “thank you” to all students of the Department of Biochemistry for many generations especially all member in my laboratory for their kind help and encouragement throughout my work.

Finally, I would like to express my deepest gratitude to my family for their unconditional love, understanding and encouragement throughout my life.

Ladda Leelawatwattana.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS AND SYMBOLS

Å	=	angstrom
AOX	=	alcohol oxidase
cDNA	=	complementary deoxyribonucleic acid
cm	=	centimeter
CSF	=	cerebrospinal fluid
^{o}C	=	degree Celcius
h	=	hour
HPLC	=	high performance liquid chromatography
kDa	=	kilodalton
kg	=	kilogram
kV	=	kilovolt
l	=	liter
M	=	molar
mg	=	milligram
min	=	minute
ml	=	milliliter
nm	=	nanometer
OD.	=	optical density
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
pmol	=	picomole
RBP	=	retinol binding protein
rpm	=	revolutions per minute
s	=	second
SDS	=	sodium dodecyl sulfate
T_3	=	triiodothyronine
T_4	=	tetraiodothyronine, thyroxine
TTR	=	transthyretin
v/v	=	volume by volume

LIST OF ABBREVIATION AND SYMBOLS (Continued)

µF	=	micro Faraday
µg	=	microgram
µl	=	microliter
µM	=	micromolar

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURES

Introduction

Thyroid hormones (THs) have a wide range of biological effects in numerous vertebrate species. They play roles in growth, development and regulation of metabolism (Brent, 1994) particular of vertebrate. In most vertebrates, thyroid gland is the only synthesis site of THs. There are several forms of THs, but the two major forms with biological activity are L-3,5,3',5',-tetraiodothyronine (L-thyroxine, T_4) and L-3,5,3',-triiodothyronine (L-triiodothyronine, T_3). T_4 functions as prohormone and exists in blood circulation as a major product of the thyroid grand. Whereas, T_3 is the biologically active form and its significant amount is obtained from the conversion of T_4 by the deiodinases system in peripheral tissue (Surk *et al*., 1973; Samuels *et al*., 1989 and Greenspan, 1994). The action and metabolism of THs occur in the intracellular compartment. Due to highly hydrophobic phenyl structure of THs, they are transported in the blood stream as a complex with plasma proteins. The binding of THs with the plasma protein carrier facilitates the transport of the THs across cell membranes (Pardridge, 1987), thus, prevents the partitioning into lipid membranes of THs, ensures the appropriate distribution of the hormones between extracellular aqueous and lipid environment and maintains the free THs pool in the blood and CSF (for reviews see Schreiber and Richardson, 1997 and Richardson, 2007).

In larger mammals, there are three major TH binding proteins found in blood. These are albumin, thyroxine binding globulin (TBG) and transthyretin (TTR). All of these proteins are synthesized in liver, except TTR is also found synthesized in choroids plexus of the brain. TTR is a homotetrameric protein (Blake *et al*., 1978) of which each subunit consists 125 to 136 amino acid residues, depending on species of the animal that TTR was isolated from. In human, the TTR subunit consists of 127 amino acid residues (Kanda *et al*., 1974). The functional tetramer of TTR contains two binding sites that differ in relative binding affinity for THs (Blake *et al*., 1978).

However, under physiological conditions, only one binding site is found occupied by TH (Page *et al*., 1973). Binding affinity of TTR to THs varied among vertebrate species. The affinity to T_4 increased while that to T_3 decreased during evolution of mammalian TTR from its ancestors similar to birds and amphibians (Chang *et al*., 1999).

The structural analysis of TTR genes from numerous vertebrate species revealed that the amino acid sequences in the central channel with THs binding site is highly conserved. The predominant changes occurred within ten and seven amino acids from the N-terminal and C-terminal ends, respectively. During evolution of vertebrates, N-terminal region of TTR is longer and more hydrophobic in avian, reptilian, amphibian and fish than in mammalian. Similar to N-terminal segment, the variation occurred leading to more hydrophobicity of the C-terminus of TTR in amphibian and fish than in mammals. In addition, the C-terminal segment of some TTRs such as pig, *Xenopus laevis* and *Rana catesbeiana* (bullfrog) is longer than the others. Due to changes in length and hydrophathy occurred at N-terminal and C-terminal regions, together with the observation that these N-terminus and C-terminus of TTR locate at the entrance of the binding sites for THs, question arise whether or not these changes in structure of N-terminal segment and/or C-terminal segment of TTR subunit affect on the accession of THs as well as other ligands to the binding site of the TTR molecule.

Apart from distribution of THs, transportation of retinol (an alcohol form of vitamin A) via binding to a retinol protein carrier named retinol binding protein (RBP) is another important function of TTR. Complex formation of TTR with RBP, has been suggested to prevent loss of vitamin A from glomerular filtration in kidney (Peterson, 1971). Binding between TTR and RBP molecules was shown involved with the hydrophobic interaction. The study by X-ray crystallography revealed that there are several amino acids that involved in the intermolecular contact of the TTR-RBP complex molecule (Naylor and Newcomer, 1999), and amino acid substitution at some positions that found in TTR variants, e.g. substitution of isoleucine to serine in TTR Ile84Ser, led to reduction in the binding affinity to RBP of TTR (Berni *et al.*, 1994).

To date, 100 point mutations in TTR gene have been identified and up to 80 of these mutations were demonstrated related to amyloidosis, a group of protein mis-folding disease. Currently, one important property of TTR i.e. acting as a cryptic protease has been reported (Liz *et al*., 2004). This proteolytic property has been studied and found associate with the occurrence of amyloidosis. The relationship between TTR proteolytic property and amyloidogenesis has been reported affect on both activation and inhibition of the amyloid fibrils formation. Proteolytic activity of TTR on apoA-I, a variant of apolipoprotein A has been proposed trigger the fibril formation of apoA-I (Liz *et al*., 2004) and reduce ability of apoA-I to promote the cholesterol efflux. Whereas, in some cases, TTR was found can act as a sequester in clearing off the amyloidogenic protein or peptides. The example of the clearing event was observed with amyloid β-protein (Aβ), a main component of amyloid plaques in Alzheimer's desease (AD). TTR was demonstrated cleave, inhibit aggregation and fibril formation of the Aβ (Golabek *et al*., 1995 and Schwarzman *et al*., 1994, 1996). In addition, a recent study showed that TTR cleaved amidated neuropeptide Y (NPY), and the cleavage of NPY by TTR was demonstrated to be related to functions of TTR to nerve cells, e.g. promoting the regeneration of neuritis (Liz *et al*., 2009). All of theses findings led to a question on a possible relationship between TTR and amyloid proteins as well as the other involving protein in physiological and pathological conditions. In addition, even though the proteolytic activity of TTR has been clarified as a serine protease (Liz *et al*., 2004), amino acid residues involved in catalysis and the underlying mechanism are still unknown.

The purpose of this research was to elucidate the role of N- and/or C-terminal segment of TTR on accession of THs and other binding ligands to the binding sites as well as the possible effect of these segments on proteolytic property of TTR, by using chimeric TTRs of which N- or C-terminal sequence is altered as tools.

Review of literatures

1. Thyroid hormone

1.1 Structure, synthesis, secretion and action of TH

THs are the amino acid derivative molecules secreted from thyroid gland which is characteristic in each vertebrate species. In mammals, the thyroid gland is composed of two lobes connected by an isthmus across the ventral surface of the trachea. In lower vertebrates such as fish, the organized thyroid gland is not formed or developed. The thyroid synthesizing cells are singled out in a small group in the loose connective tissue of the pharynx (for review sees Power *et al*., 2000).

The basic molecular structure of TH can be divided into three regions: a hydrophilic 4′-hydroxyl group, two hydrophobic phenyl rings linked together by an ether bond, and an amino acid side chain on the tyrosine ring. Iodide substitutions to hydrogen atoms on the phenyl rings generate several forms of TH but the major forms with significant biological activity are T_4 and T_3 (Figure 1.1) (Samuels *et al*., 1988). Thyroid gland secretes both T_4 and T_3 to the blood circulation. However, T_4 is the major form of the hormone being secreted, and functions as a prohormone. The conversion into the active molecule, T_3 (approximately 80%) occurs in the peripheral tissue by deiodination so that one of iodide atom is removed from the outer ring of T_4 (Eale, 1984 and Bianco *et al*., 2002). Deiodination is the pathway that produces not only the active metabolite, i.e. T_3, but also the less or inactive metabolites by removal of an iodine atom on the inner ring. The example of such removal is the conversion of T_4 to form 3,5,5′-triiodothyronine (reverse T_3 or rT_3) or the conversion of the active triiodothyronine to the inactive diiodothyronine (T_2) (Figure 1.1) (Orozco *et al*., 1997). The concentration of T_4 in human serum and daily production rate in the thyroid grand are 8.6 µg/dL and 130 nmol/day/70 kg, respectively (Chopa, 1976). The turn over rate of T_4 in human is about 10% per day (for review see Utiger, 1995). While concentration of T_3 in human serum ranges from 110 to 180 ng/dL, and with the turn over rate of 75% per day (Utiger, 1995; for review sees Chopa, 1996). Since T_3 has higher affinity to TH receptor and it has more potent in TH action than T_4, therefore, it has been considered to be the major active form of TH in target tissues,

while T_4 is the transport form (Gross *et a*l., 1952; Lerman 1953; Surks and Oppenheimer, 1977, for review see Richardson, 2007).

The synthesis and secretion of THs are regulated by a negative feedback of hypothalamus-pituitary-thyroid gland network (for review see Yen, 2001). The synthesis site for THs in the thyroid gland locates in the highly vascularized spheroidal follicles, consisting of a layer of epithelial cells surrounding a lumen filled with proteinaceous colloid. The THs require iodide ion (I^-) and an iodoperoxidase (or thyroid peroxidase) involved in their synthesis. Iodides are trapped from the bloodstream, then iodinated and coupled by iodoperoxidase to tyrosine residues in a protein called thyroglobulin to form the iodinated thyroglobulin (Ohmiya *et al*., 1990; Palumbo *et al*., 1990; Hayashi *et al*., 1991 and Xiao *et al*., 1995). Formation of T_4 and T_3 occurs within the thyroglobulin molecule, involves an oxidative coupling mechanism of diiodothyronine and/or monoiodothyronine to a free radical and, then, interaction of two radicals (Harington and Rivers, 1945). Hydrolysis to release T_4 and T_3 from the thyroid gland into the bloodstream simultaneously takes place as the thyroid follicles incorporate to lyzosomes. Then the T_4 and T_3 in the blood are distributed to the peripheral tissues via TH distributor proteins.

THs have multiple levels of control but their predominant action exerts via the transcriptional pathway (Glass and Holloway, 1990). The action at this level is mediated through thyroid hormone receptor (TR) and its isoforms, which functions as the hormone-activated transcription factor, and consequently controls the expression of the target genes (Zhang and Lazar, 2000). The mechanism process of TH action at genomic level via TR in target cells was summarized in Figure 1.2. T_4 and T_3 enter the cell by both passive and facilitate diffusions. T_4 is, then, deiodinized to an active metabolite, T_3, in the cytoplasm of the target cell. T_3 binds to TRs that are constitutive bound to thyroid hormone responsive elements (TREs) of target gene as heterodimer or possibly homodimer form. The T_3-TR complex further interacts with other specific co-activators at DNA regulatory region resulted in increase of gene expression and products of the target gene.

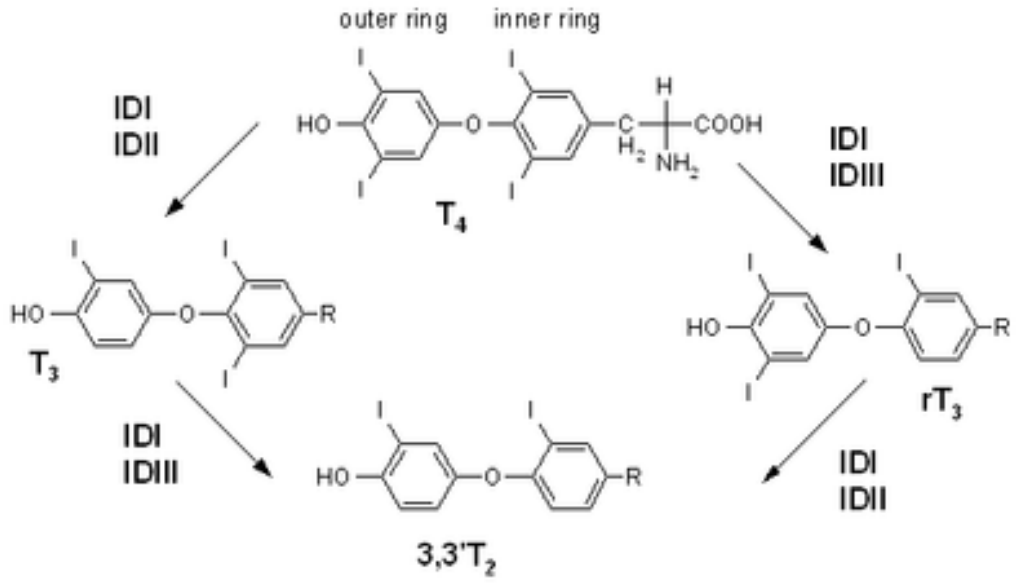


Figure 1.1 Structure of several types of THs

The major TH that secreted from thyroid gland is T_4, which can be converted to an active form T_3 via a system of deiodination mostly in the target tissues, by deiodinase type I (IDI) and type II (IDII). In addition, T_4 and T_3 can be converted or metabolized to an inactive forms, rT_3 and $T_{2,}$ by IDI and deiodinase type III (DIII).

(Source: http://en.wikipedia.org/wiki/Triiodothyronine, 23/01/09)

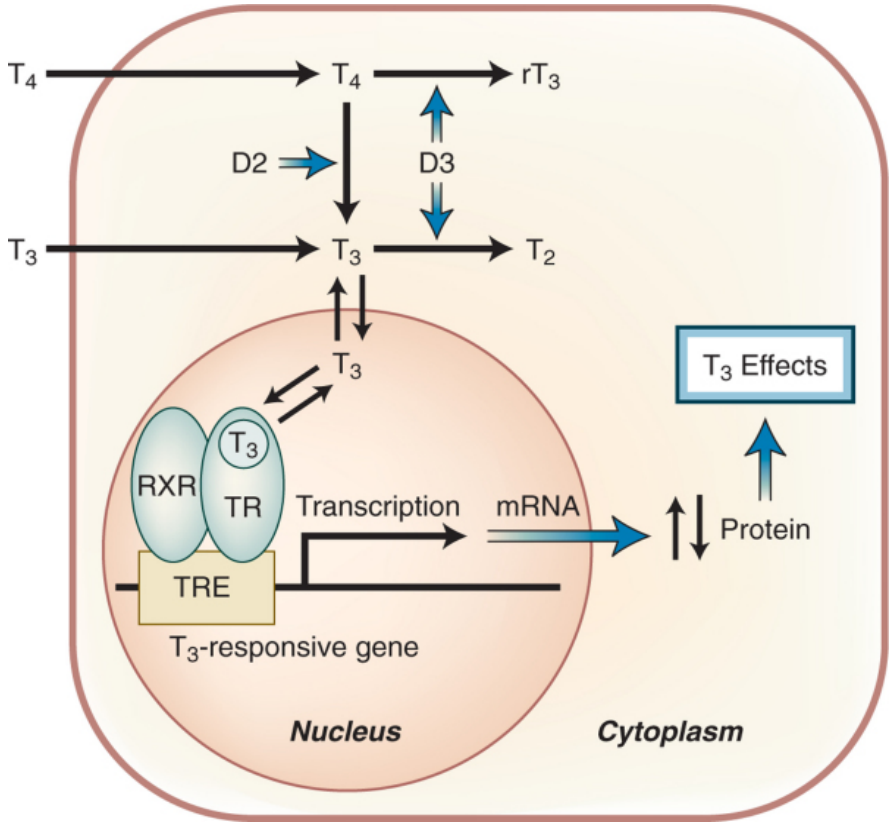


Figure 1.2 Mechanism of genomic action of THs.

(Source: http://mdconsult.com/das/book/body/0/0/1555/I4-u, 23/01/09)

1.2 Distribution of TH and the TH distributor proteins

TH actions on metabolisms occur in the intracellular compartment. However, because of their highly hydrophobic phenyl structure, THs have a strong tendency to partition into lipid membranes (Hillier, 1970 and Dickson *et al*., 1987) that can result in marked depletion of the hormones from blood by permeation into cells (Mendel *et al*., 1987). Thus, a mediator for moving the hormones across the plasma membrane is required to ensure distribution to the peripheral tissues those locate far from the thyroid gland. There are three TH distributor proteins (THDPs) found in the bloodstream of higher vertebrates. They are thyroxine-binding globulin (TBG), TTR and albumin. In human, 99.97% of T_4 and 99.7% of T_3 is bound to these THDPs (Mendel *et al*., 1989). The name "thyroid hormone distributor proteins" has been coined due to role of these three proteins in ensuring distribution of THs through out the tissues and maintaining sufficient amount of the hormones circulating in blood and cerebrospinal fluid (CSF) (for review see Richardson, 2007). All vertebrate species possess at least one of these THDPs (for review see Schreiber and Richardson, 1997 and Schreiber *et al*., 1998). It has been suggested that these three THDPs form a buffering system for free T_4 in plasma that resulted to the protection against hypothyroidism and hyperthyroidism (Schreiber and Richardson, 1997).

In human, approximate 20%, 11% and 68% of T_4 and 27%, 38% and 35 % of T_3 are bound to albumin, TTR and TBG, respectively (for review see Schreiber *et al*., 1995). In the lower vertebrates such as amphibians and fish, little is still known about these proteins, however functions of albumin as the major T_4 binding protein and TTR as the major T_3 binding protein have been suggested (for review see Power *et al*., 2000).

TBG is a minor component of the α-globulin fraction in serum. It is a glycoprotein with a molecular weight of 54 kDa. The gene coding for this protein locates on the long arm of X chromosome. The primary structure of TBG is very conserved (Frink *et al*., 1986; Imamura *et al*., 1991 and Tsykin and Schreiber, 1993). In human, TBG present in the plasma at lowest concentration (15 mg/L) in comparing to TTR (300 mg/L) and albumin (45,000 mg/L) (for review see Power *et al*., 2000). However, it carries about 75% of all T_4 in plasma as a consequence of its extremely high affinity (K_{TBG} = 1.7x 10^{10} M^{-1}, K_{TTR} = 2.3x 10^{8} M^{-1} and K_{alb} = 6.2 x 10^{5} M^{-1})

(Woeber and Ingbar, 1968). Nevertheless, the binding capacity of TBG for T_4 and T_3 is low in comparing to those of TTR and albumin (for review see Power *et al*., 2000). This protein is found in plasma of large eutherians including human, monkey, cattle sheep, goat, buffalo, horse, swine and dog, but it was not observed in plasma of adult rat (Larsson *et al*., 1985 and Richardson *et al*., 1994).

All of these three THDPs are synthesized by liver and secreted into the plasma. Only TTR was found is also synthesized in brain and the TTR in brain was demonstrated is involved in the transport of TH from blood to brain (Dickson *et al*., 1987; Scheiber *et al*., 1990; Chanoine *et al*., 1992 and Southwell *et al*., 1993). TTR is synthesized in liver of eutherians, diprotodont marsupials and bird during development and adult stages of the animals. However, it was not found in adult Australian polyprotodont marsupials, monotreme, reptile, amphibian and fish (Richardson *et al*., 1994). In amphibian, TTR was found in liver only in a specific stage of life i.e. during metamorphosis (Yamauchi *et al*., 1998 and Prapunpoj *et al*., 2000a). Moreover, TTR is the major THDPs in the brain of amphibians, reptiles, birds and mammals (Harms *et al*., 1991; Tamauchi *et al*., 1993 and Richardson *et al*., 1994).

Albumin is a single polypeptide with molecular weight of 65 kDa. It is the oldest and the most widely distributed THDP in plasma of vertebrates. In some species such as fish, amphibians, reptiles and mammals, albumin has been demonstrated to be the major THDP (Richardson *et al*., 1994). In human, albumin can bind to THs with the lowest binding affinity in comparing to TTR and TBG, therefore, it is present in high amount in plasma (for review sees Power *et al*., 2000). The early appearance of albumin gene expression during evolution, its widely distribution among species and the conservation of its amino acid sequence, make albumin to be one of the important THDPs though disruption of the albumin gene in human and rat was shown no effect on health or impairment of a euthyroid status (Richardson *et al*., 1994).

1.3 Role of THs

THs exert many effects on growth, development and metabolism of higher vertebrates, e.g. mammals and birds, and lower vertebrates, e.g. amphibians, reptiles

and fish. The influences of THs on growth mediated through the regulation of growth hormone synthesis have been documented in children and young animals (Brent, 1994). In contrast, the effect of THs on developmental processes is very important during life of non-mammalian vertebrates. In amphibians, the ontogenic transformation involving changes in specificity and remodeling of tissues and organs, e.g. neuron system and gastrointestinal tract, that occur during the transition from larvae to juveniles and to adult is induced and regulated by THs (for reviews see Hourdry 1993; Tata 1993; Su *et al*., 1999 and Shi *et al*., 2001). THs also play an important role in development and differentiation during metamorphosis (smoltification) in fish (Yamano *et al*., 1991 and Miwa *et al*., 1992).

For higher vertebrates, in particular human, a deficiency of the hormones (hypothyroidism) results in severe impairment of mental development and growth in childhood (cretinism). While, TH deficiency in adults leads to a decrease in basal metabolic rate, lower body temperature and heart rate, which results in weight gains and a series of symptoms called “myxedema”. In contrast, an increase in TH levels (hyperthyroidism) leads to an increased metabolic rate and body temperature, and weight loss. In lower vertebrates, gluconeogenesis and glycolysis as well as lipogenesis and lipolysis are the major metabolic pathways affected by actions of TH. Short-term effect of T_3 on the level of malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH) and β-hydroxy-β-methylglutaryl CoA reductase (HMGCoAR) was detected in the Southeast Asian climbing perch, *Anabas testudineus* (Varghese, 1999 and Tripathi and Verma, 2003).

2. Transthyretin (TTR)

2.1 History of TTR

Transthyretin or TTR was first identified in 1942 by Kabat *et al*. in human cerebrospinal fluid (CSF) and thereafter in human serum by Seibert and Nielson (1942). At first, it was called component X (Gavrilesco *et al*., 1955). However, as it was the only protein that migrates faster than albumin, so it was later named prealbumin (PA). In 1958, PA was found binding to THs. “PA” name was thus changed to thyroxine binding prealbumin or TBPA. Thereafter, in 1969, Raz and

Goodman found that TBPA could also bind retinol binding protein or RBP. So, finally, in 1981, the name TBPA was changed again to transthyretin which explained roles of this protein in which it was the THs and RBP transporter. This name has been used until now.

TTR is a globular protein with four identical subunits (Brance *et al*., 1971 and Blake *et al*., 1978). These subunits organized to form tetrahedral structure of TTR and providing two binding sites for THs, however, only one binding site is occupied by the hormone under physiological condition due to negative co-operativity effect (Page *et al*., 1973 and Nilsson *et al*., 1975). Each subunit of TTR composed of 125 to 136 amino acid residues depending on animal species that TTR was isolated from. In human, each subunit of TTR compose of 127 amino acids with mass of 13,745 daltons (Kanda *et al*., 1974) leading to 54,980 daltons of the TTR tetrameric molecule. In nature, tetramer of TTR is not modified by phosphorylation, glycosylation or acylation though it has possible sites for modification.

2.2 Three-dimensional structure of TTR

To date, only TTR tetramer from 4 vertebrates other than human have been crystallized and three dimensional structure had been revealed by using molecular replacement techniques in comparison to the structure of human TTR, these are from rat (Wojtezak, 1997), mouse (Reixach *et al.*, 2008), chicken (Sunde *et al*., 1996) and seabream (Eneqvist *et al*., 2004). Human TTR was the first protein whose amino acid sequence was determined from its crystal structure. Blake and coworkers determined the three dimensional structure of wild-type human TTR, originally at 2.5Å resolution in the year 1974 and then refined to 1.8Å in four years later (Blake *et al*., 1978). The primary structure of human TTR composed of 127 amino acid residues (Kanda *et al*., 1942). The X-ray crystallography revealed that this polypeptide chain interacts to form the secondary structure with dominantly β-pleated sheets. Figure 1.3 and 1.4 show the tertiary structure of TTR. From the ribbon structure of TTR monomer, 45% of the amino acid of TTR monomer are organized into eight β-sheets which form into two four β-sheet strands, denoted as DAGH and CBEF. All strands, except strand A and G, interact antiparallelly to each other. The residues that located between these two sheets are almost exclusively hydrophobic and constitute the core of the subunit.

Moreover, the single short α-helix (about 5%), composed of nine residues, comes after strand E. The rest of amino acid residues are organized into seven loops connecting the eight β-strands as well as the N- and C-terminal segments which locate outside the core structure of the tetramer. It is only the ten and five amino acid residues at N- and C-termini, respectively, are not involved in the folding, act as a head and tail of the TTR molecule. From X-ray crystallography, the N-terminal region of TTR had a semi-circular rod structure, overhangs at the entrance to the central channel of the TTR tetramer (Hamilton *et al*., 1993). The quaternary structure of TTR (Figure 1.5) formed by two types of interaction: dimer that arises from monomer-monomer interaction and tetramer that is formed from the linkage of the two dimers. Dimers of TTR formed by edge to edge interaction of two monomers that results in pair twisted eight stranded β-sheets, one inner DAGHH'G'A'D' and one outer CBEFF'E'B'C'. The dominant interaction that stabilize each dimer are hydrogen bonding between the edge of strand F and H of the two β-sheet of each monomers (between F and F′, and H and H′) and also two complex hydrophobic interactions and two water bridges (Blake *et al*., 1978). The tetramer formation of TTR is derived from interaction of two dimers. The amino acid residues that are involved in this contact locate at the edge of strands A and B, which form a loop structure called AB loop, and at the H strands of the opposite dimer. The interactions that occur along the edge of the sheets are hydrophobic interaction and hydrogen bonding. However, there is no interactions occur in the centre of the sheets. This leads to the formation of a cylindrical channel that run through the centre of the TTR molecule where binding sites for THs exist. Due to the overall structure of TTR that comprised of very high content of β-sheet structure, the interactions between monomers to form dimers and between dimers to form tetramer, TTR is one of the most stable globular protein (Branch *et al*., 1971 and 1972).

As the consequence of tetrahedral arrangement of the four identical subunits of TTR, a central channel with 50 Å in length and 8 Å in diameter is formed. The two binding sites for THs which differ in their relative binding affinities locate in this central channel (Figure 1.6A). The amino acid residues that are involved in the ligand binding are located in strand A (residue14-18), D (residue 53-56), and G and H (residue 105-122) (Neuman *et al*., 2001). The chemical nature of the channel consists

of three main elements. First is a hydrophilic centre forming from the hydroxyl group of Ser112, Ser115, Ser117, Thr119 and associated bound water molecules. Second is a hydrophobic patch formed by methyl groups of Leu17, Thr106, Ala108, Leu110 and Val121. A group of charge residues such as Lys15, Glu54 and His56 constitute the third element at the entrance to the channel (for reviews see Blake *et al.*, 1981 and Hamilton and Benson, 2001). In the binding pocket (figure1.6B), T_4 fits to the central channel via the contact between the components of the hormone and the protein environment. The 3′ and 5′ iodine interact with the side chain of Leu17 and Leu110 and lie within the pocket lined with methyl groups. The associate water molecules facilitate the 4 hydroxyl group to interact with the hydroxyl group of Ser117 and Thr119. Finally, the α-carboxylate and α-amino groups point outside the channel so that the contact is made with the charged residues His56, Lys15 and Glu54 at entrance of the channel as shown in figure 1.6B (Oatley *et al*., 1982 and 1984 and Hamilton and Benson, 2001). It is not only the THs that can interact at the binding sites of TTR that locate in the central pocket, but several compounds including drugs, metal ions, plant flavonoids and industrial pollutants such as polychlorinated bisphenyls (PCBs) also bind TTR at the internal binding sites (Köhrle *et al*., 1988; Munro *et al*., 1989; Craik *et al*., 1996 and Zheng *et al*., 2003). Binding of some of these compounds affected on TH metabolism as they compete with THs for binding to TTR, therefore affect on normal level of TH in plasma, which subsequently lead to diseases such as hypothyroidism. On the contrary, some compounds can increase stability of TTR tetramer and protect the molecule from dissociation, which is a crucial step of TTR aggregation leading to the disease called amyloidosis (Miller *et al*., 2004). The basic structural study of the T_4 binding in the presence of T_4 analogues demonstrated various possible binding modes of TTR, including forward and reverse modes. In the forward mode of the binding, the carboxyl group of T_4 or its analogues situates at the outer part of the binding cavity and make interaction with side chain of Lysine15 (as showed in Figure 1.6B). In contrast, turning into the opposite direction to that in the forward mode of the interacting groups in the binding pocket of TTR was proposed for the reverse mode (De La Paz *et a*l., 1992). The accession of T_3 and T_4 to the TTR binding site has been proposed as an mix mode. For T_3, a combination of 45% forward mode and 55% reverse mode or a variety of forward binding modes is

possible. While T_4, a combination of forward and reverse modes was proposed but the proportion is still unknown. Blake and colleague suggested that the remarkable ability of TTR to accommodate ligands in a variety of binding modes may reflect its function as a transport protein for THs and their deiodination products (De la Paz *et al*., 1992) which differ in their conformation flexibility at the ether link and in the charge properties of the phenolic hydroxyl group under physiological conditions (for review see Hamilton and Benson 2001).

The dimer of TTR has been proposed to be the basic unit of the TTR tetrameric structure. This was supported from the presence of the TTR dimer in the SDS-PAGE analysis even under denaturing condition (Robbin *et al*., 1978). The dimer was not dissociate although boiling for 5-10 min in the presence of SDS or urea. The TTR tetramer is stable within pH range of 3.5-12 and no dissociation was observed in strong acidic or alkaline. Dissociation of the TTR tetramer only occurred by a prolong exposure to 6M guanidinium-HCl (Branch *et al.*, 1972). At least 80% of human TTR tetramer was still intact after boiling for 20s in the presence of 1%SDS and 10mM β-mercaptoethanol (Bellovino *et al*., 1998). Moreover, TTR lost the binding capacity to T_4 when exposed to 6 M urea, however, the binding was restored after the urea was removed from the protein (Raz and Goodman, 1969).

2.3 Genomic structure of TTR

For genomic structure of TTR, it was demonstrated that the human TTR is a single copy gene localizes on the long arm of chromosome18 (Sparkes *et al*., 1987) and spans to 6.9 kb. The gene coding for TTR composed of four exons and three introns. A TATA box-like sequence locates at nucleotide -24 to -30 and the CAAT box-like sequence locates at nucleotide -95 to -102 (Sasaki *et al*., 1985; Tsuzuki *et al*., 1985: for review see Power *et al*., 2000). The first exon of TTR gene encodes for the 5′ untranslated region, the 18 amino acids of signal peptide, and 3 amino acid residues of the mature protein. Exon2, 3 and 4 encodes for residues 4 to 47, 48 to 92 and 93 to 127, respectively, and these are corresponding to 44, 45 and 35 amino acid residues, respectively. A polyadenylation signal sequence, AATAAA was situated 23 base pairs upstream from the polyadenylation site (Tsuzuki *et al*., 1985). The three introns span for 934, 2090 and 3308 bp, respectively. These parts of TTR gene was proposed

to involve in expression or regulation of the gene since the nucleotide sequences that homologous to human Alu-type repeat sequence in the second and the third intron were detected in this region. Furthermore, two unidentified open reading frames (ORFs) with the same transcription direction as TTR and putative consensus regulatory sequences for transcription of the first and the third intron were located in this region. Although, the true function of these sequences is unknown, a function in encoding protein for TTR gene expression regulation or mRNA editing has been proposed (Tsuzuki *et al*., 1985).

Figure 1.3 Structure of TTR monomer

A monomer of TTR comprises of two strands of four β-sheets, which denoted as DAGH and CBEF, and a short α-helix that runs after strand E (Hamilton *et al*., 1993 and Armen *et al*., 2004)

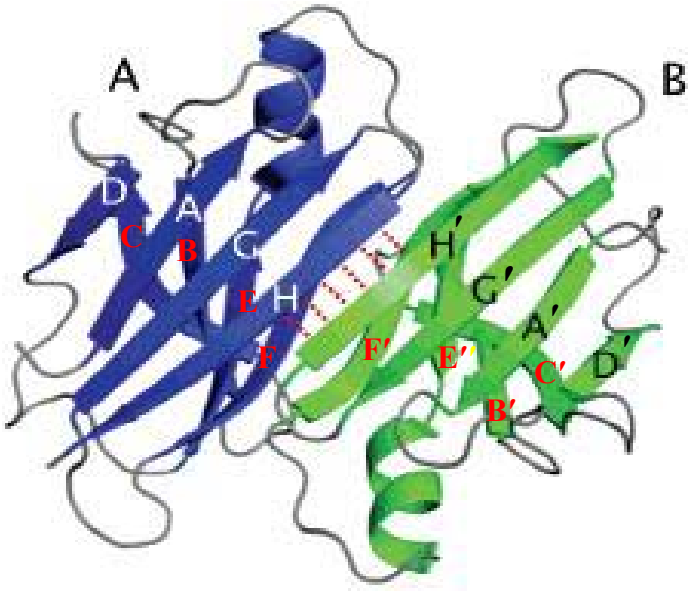


Figure 1.4 Structure of TTR dimer

The edge to edge β-sheet interfaces between two TTR monomers generate a TTR dimer. The red dashes represent the hydrogen bonding interface between H-strands of A and B subunit.

(Modified from Foss *et al*., 2005).

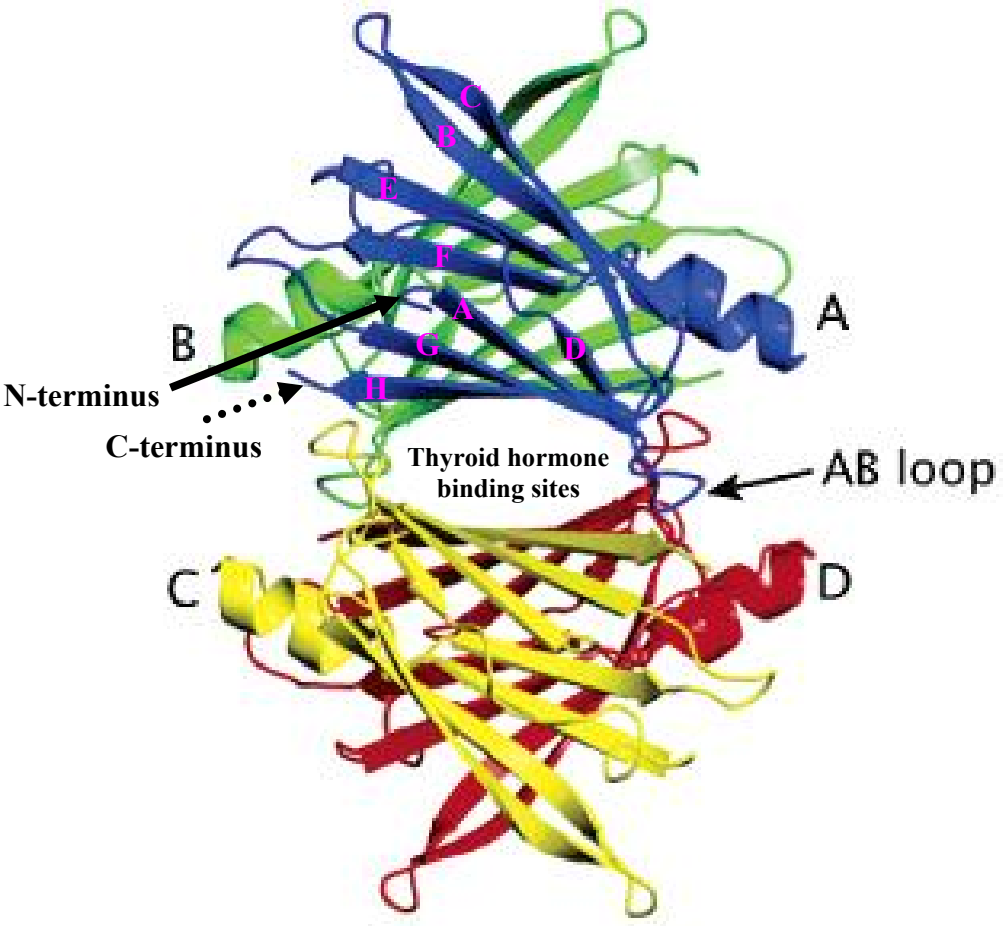


Figure 1.5 Ribbon diagram of the TTR tetramer

Four monomers of TTR, which are different in color, interact to form two dimers. The two dimers are further linked through AB loop to form a tetramer. The central channel where the binding sites for THs and other ligands exist is generated from the interaction of the two dimers. The N- and C-termini (four of each) those locate at entrance to the central channel were indicated by arrow and broken arrow, respectively. (Modified from Foss *et al*., 2005)

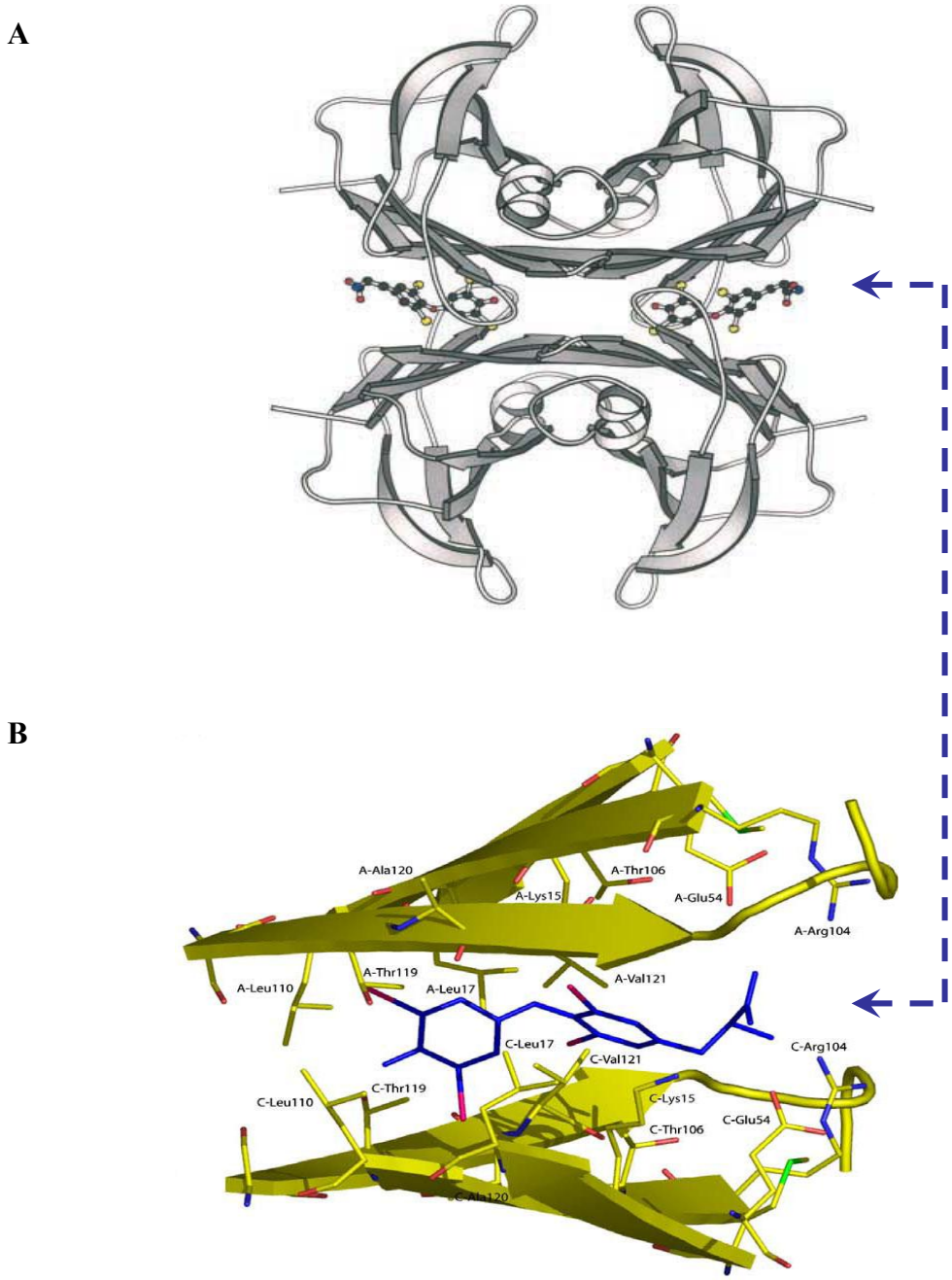


Figure 1.6 Binding of TH to the TTR binding site

A)The ribbon model of TTR tetramer showed the binding of TH molecule in the two possible binding sites of TTR, though there is a negative co-operativity between the two binding sites.

B)The TH molecule that entries to the binding site make contact with side chains of the amino acids that locate in the central pocket (Hamilton and Benson, 2001 and Lundberg *et al*., 2006).

2.4 Site of TTR synthesis and secretion

Similar to other plasma proteins, TTR is synthesized and secreted into bloodstream by liver (Dickson *et al*., 1982). Once TTR is synthesized in hepatocytes, its signal sequence, locates at the N-terminus, is cleaved during translocation into an endoplasmic reticulum (ER). Within the ER, the TTR monomer is assemble into tetramer prior to being secreted (Bellovino *et al*., 1996). In general, TTR levels in plasma increase gradually after birth until they reach the concentration of 20-40 mg/dl in adult, then it decrease after the fifth decade of life (Stabilini *et al*., 1968; Benvenga *et al*., 1986; for review see Hamilton and Benson 2001)**.**

The other major synthesis site of TTR is the brain. By dot blot hybridization and Northern analysis of the RNAs extracted from various region of rat brain revealed strong signals from choroid plexus. Furthermore, the microscopic *in situ* hybridization demonstrated that the TTR mRNA in the choroids plexus locate in the epithelium cells lining to the barrier separating the brain from the blood (blood-brain barrier or BBB) (Dickson *et al*., 1985; for review see Schreiber 2002). In human brain, abundant of TTR mRNA was detected in choroids plexus (Dickson and Schreiber 1986; Herbert *et al*., 1986). TTR was synthesized in the epithelium cell of choroids plexus prior it was secreted into cerebrospinal fluid (CSF) (Southwell *et al*., 1993). In comparison to that of albumin and other plasma protein, the concentration of TTR is much higher in CSF than in blood. Approximate 50% of the secreted proteins and about 12% of the newly synthesized proteins in choroids plexus are TTR (Dickson *et al*., 1986; for review see Schreiber 2002). Although the *de novo* synthesis of TTR by choroid plexus has been demonstrated in human, ~10% of the TTR in CSF was derived from plasma (Reiber, 1997). Besides the liver and the brain, minor synthesis of TTR has also been identified in visceral yolk sac and placenta (Soprano *et al*., 1986), retinal and ciliary pigment epithelium (Cavallaro *et al*., 1990), human intestine during development (Loughna *et al*., 1995), pancreases in islet of Langerhans (Kato *et al*., 1985) and menings (Blay *et al*., 1993).

The synthesis of TTR in liver is a negative acute-phase response. The synthesis rate and level of TTR in blood decreased markedly during condition of malnutrition or chronic inflammation (Schreiber and Howlett, 1983). Although, the synthesis of TTR in the liver altered because of stresses, the synthesis in the choroid

plexus was very slightly affected (Dickson *et al*., 1982). This indicated that the TTR gene transcription in the choroids plexus is not under negative acute phase regulation and it implied that synthesis of TTR in choroids plexus is independently regulated from the TTR gene in liver (Dickson *et al*., 1986; for review see Richardson, 2007).

In rat, it has been demonstrated that liver, kidney, muscle and skin are the major site of TTR degradation and the degradation of TTR does not take place in the nervous system (Makover *et al*., 1988; for review see Palha, 2002)

2.5 Functions of TTR

There are two major functions of TTR that have been suggested: a transporter for THs and for retinol in which the latter via binding to RBP.

2.5.1 TTR as a TH transporter

TTR has been first described binding to T_4 and T_3 in 1958 (Ingbar, 1958). In human serum, about 15 % of T_4 was bound to TTR whereas up to 80% of the hormone in central nervous system was transported by this protein (Herbert *et al*., 1986)

From the early studies, it concluded that TTR had only one site for T_4 (Nillson *et al*., 1975 and Robbin, 1976). However, by using 8-anilinonapthalene-1-sulfonate, a fluorescence probe, it showed that TTR poses two sites for T_4, one with high affinity ($K_d = 1.0x10^{-8}\ M^{-1}$) and a second site with lower affinity ($K_d = 19.5x10^{5}\ M^{-1}$) (Ferguson *et al*., 1975 and Cheng *et al*., 1977). The difference of this association constant is explained by the negative co-operativity effect of ligand binding that lead to conformational change of TTR molecule and then affect to the binding affinity of second ligand molecule (Neuman *et al*., 2001). The structural basis for the negative co-operativity effect in TTR was studied by comparing diameters of the binding channel of two types of TTR i.e. apoTTR and TTR complexes with ligands. This demonstrated that the channel diameter of the first was larger than the second site. The binding of TH to the first ligand binding site changed diameter of the second site by increasing the diameter of the binding channel of the second site, in both outer and inner part of channel. As a consequence, the binding site collapsed (Neuman *et al*., 2001) and led to looser bound of the second hormone.

Brain is an important target organ for THs (Shambaugh, 1986). This organ is separated from the bloodstream by the blood-brain and blood-cerebrospinal fluid barrier (CSF) (Schreiber *et al*., 1990). Since these barriers do not allow THs, an amphipathic molecule, to penetrate therefore a necessity of THs carrier or distributor was suggested. The finding of high level of TTR mRNA in choroid plexus together with the finding that the synthesized TTR was secreted to CSF leading to a hypothesis that the transport of TH from blood to brain might involve with TTR in choroid plexus and CSF (Aleshire *et al*., 1983; Dickson *et al*., 1987; Schreiber *et al*., 1990 and Chanoine *et al*., 1992). The mechanism model for T_4 transport from blood to brain was presented by Southwell *et al*. (1993), as shown in Figure 1.7. In the blood, the T_4 that bound to the TH distributor proteins (THDPs) is in equilibrium with the free T_4, which can enter the brain across the choroid plexus epithelium cell. In moving across the membrane, the T_4 bound to newly synthesized TTR in choroid plexus or to TTR that is secreted to CSF, leading to a movement of T_4 from choroids plexus to the CSF down the free T_4 gradient. T_4 also can enter the brain through cerebral endothelial cell. However, as the free concentration of T_4 in CSF is higher than that in blood, it can not account for the net movement of T_4 from the blood into the CSF (Southwell *et al*., 1993; for review see Richardson, 2007). Although in this model, T_4 was shown passing through CSF in both free and bound forms with TTR, but Richardson (2007) proposed that TTR secreted by the choroid plexus enters the CSF primarily as a T_4-TTR complex.

Moreover, as already mention above that TTR in the choroids plexus is not under negative acute phase regulation, so that the function of TTR in the brain has been proposed to protect the brain from hypothyroidism during trauma and inflammation.

2.5.2 TTR as a retinol (vitamin A) carrier via binding to retinol binding protein (RBP)

Besides functioning as a transporter for THs, TTR also acts as a carrier of retinol from the storage site in liver to various target tissues (Blomhoff 1994; Sivaprasadarao and Findlay, 1994; for review see Raghu and Sivakumar 2004). In plasma, retinol is bound to a specific protein named retinol binding protein or RBP that, in turn, forms a complex with TTR. RBP was first isolated by Kanai and

coworkers in 1968. The protein is a single polypeptide chain protein of 182 amino acid residues with a molecular weight of 21 kDa (Goodman, 1984). The X-ray crystallography of the RBP polypeptide chain revealed that RBP comprised of eight strands of anti-parallel β-sheet forming a deep hydrophobic cleft in the molecule, which followed by a short alpha helical segment.

Retinol binds RBP in this hydrophobic clef to the position that the cyclohexin ring is buried inside the protein but allowed its polar hydroxyl group pointing out to the outside on the surface of the molecule (Newcomer *et al*., 1984; Zanotti *et al*., 1993; Monaco *et al*. 1994 and 1995 and Raghu *et al*., 2004). It has been proposed that the binding of RBP to tetrameric TTR under physiological conditions prevented loss of RBP-retinol complex (21 kDa) through glomerular filtration of the kidneys since the apparent molecular mass of the TTR-RBP/retinol complex or retinol/RBP-TTR-RBP/retinol complex increased to ~80-100 kDa, respectively (Raz and Goodman, 1969). In human plasma, about 40% of TTR circulates as a complex with RBP whereas less than 1% circulates as a complex with T_4 (Woeber and Ingbar, 1968). The bindings of T_4 and RBP to TTR are independent, while the binding of retinol to RBP seem to have a positive effect on protein-protein interaction (Goodman and Raz, 1972 and Jaarsveld *et al*. 1973). The complex formation of TTR and RBP was found involved with hydrophobic interaction (Peterson, 1971) and the highest binding occurred near physiological pH. The binding gradually decreased at above or below the neutral pH (Jaarsveld *et al*. 1973). The TTR-RBP complex dissociated readily at low ionic strength and in the presence of 6 M urea. By using many techniques, it revealed that the TTR binding site for RBP located on the surface of TTR molecule, and one TTR molecule has up to four possible binding sites for RBP. However, because of the steric hindrance between ligands, up to two binding sites of TTR can be occupied (Monaco and Coda, 1995 and Raghu *et al*., 2003). However, the *in vivo* studies revealed that TTR and RBP form complex with a 1:1 molar ratio (Raghu *et al*., 2003) due to the limiting concentration of RBP in the plasma.

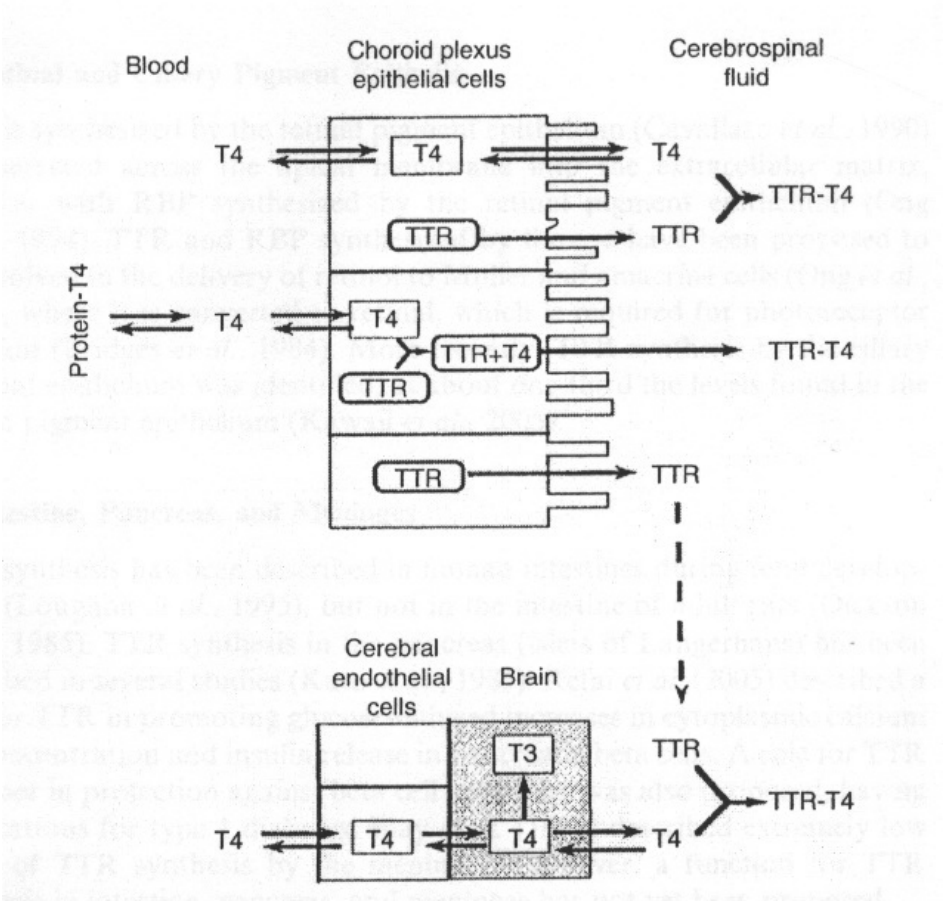


Figure 1.7 Model for TTR mediated T_4 transport into the CSF through choroids plexus

In this hypothetical route, T4 can enter the brain by two ways i.e. by moving across the choroids plexus epithelium cell (upper) or by moving across the cerebral endothelium cell (lower) (Southwell *et al*., 1993, for review see Richardson, 2007).

The crystal structure of two TTR–RBP complexes, the human TTR-chicken RBP (Monaco *et al*., 1995) and the human TTR-human RBP complex (Naylor and New comer, 1999) were revealed. The complex that observed in both crystals was the hexamer to which two molecules of RBP bound one molecule of TTR. However, the quaternary structure of these two complexes was different. In the human TTR-chicken RBP complex, the two molecules of RBP formed inter molecular contacts with the same TTR dimer (Figure 1.8) and additional contacts of each RBP with one of the other two monomers are determined. In contrast, two RBP molecules interacted to the opposite dimers of the TTR tetramer was observed in the homologous TTR-RBP complex (figure1.9). The main amino acid residues that involved in the intermolecular contacts between TTR and RBP molecules in these two complexes are shown in Table 1.1. All of these amino acids, except five, are similar in both complexes (Monaco, 2000). In the homologous TTR-RBP complex of human TTR, Arg21, Val20, Leu, 82 and Ile 84 from two monomer of TTR participated in the intermolecular contacts with Tryp67, Phe 96, Leu 63 and Leu97 of RBP. The amino acids from RBP that involved in the intermolecular contact locate at the entrance of the β barrel where the oxygen from retinol hydroxyl group is located. In addition, the C-terminus, which is disorder in the structure, of RBP was found nestle in a hydrophobic patch at the interface of TTR-RBP, however, only in one of the two monomers (Hamilton and Benson, 2001).

Recently, the mutation of human TTR gene that leads to a disease called amyloidosis have been widely studied (for review see Saraiva, 2002). Among the reported mutations, three are participated in the interactions of TTR with RBP. These included the substitution of Tyr at position 114 by Cys (Ueno *et al*., 1990), the substitution of Val at position 20 by an Ile (Jacobson *et al*., 1997) and the substitution of Ile at position 84 by Ser (Benson *et al*., 1983). The association constant of the binding between TTR Ile84Ser and normal RBP is negligible, which was resulted to reduction of the RBP concentration in the plasma of these patients (Berni *et al*., 1994). The *in vivo* effect of mutation in Tyr114Cys and Val20Ile still has not been reported.

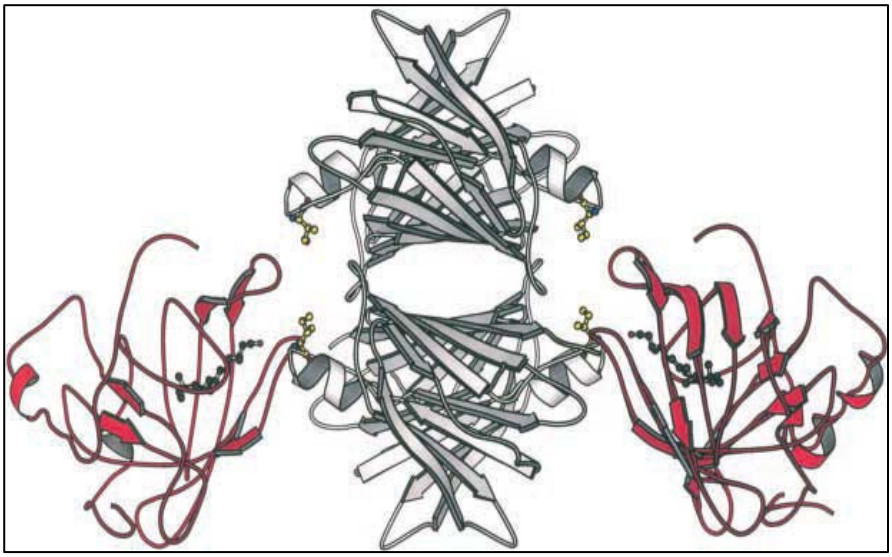


Figure1.8 Three dimensional structure of chicken RBP and human TTR complex

The human TTR molecule is shown in grey with the position of residue 84 in yellow. The two RBP molecules that interact to TTR are indicated in red and the retinol molecule is in black. This drawing was generated by MOLSCRIPT using file 1RLB (Monaco *et al*., 1995 and Hamilton and Benson., 2000).

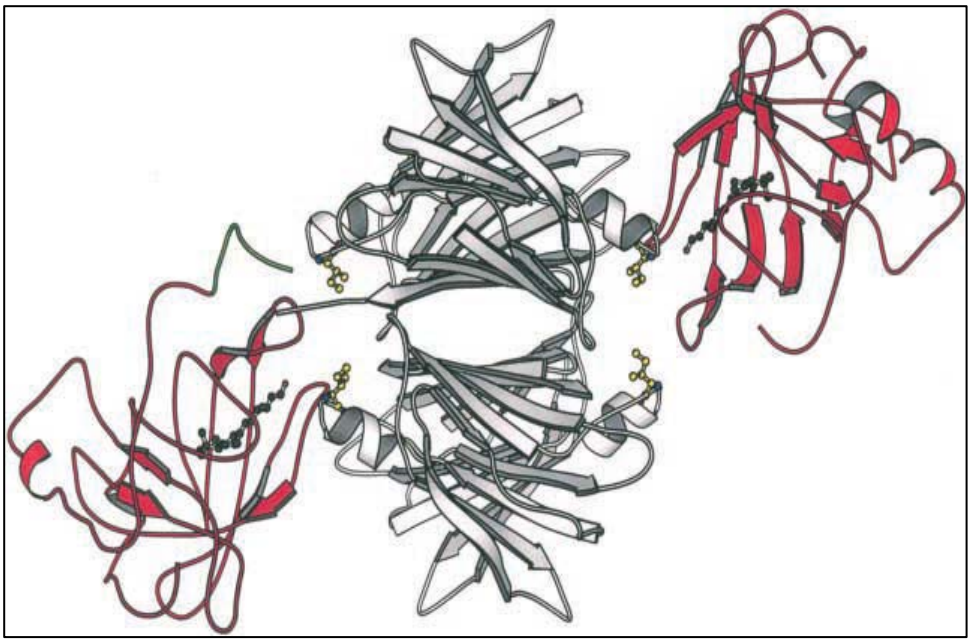


Figure 1.9 The human TTR-RBP complex

The human TTR molecule is shown in grey with the position of residue 84 in yellow. The two RBP molecules that interact to TTR are indicated in red with the C-terminal segment that involved in the binding in green. The retinol molecules are in black. This drawing was generated by MOLSCRIPT using file 1QAB (Naylor and Newcomer., 1999 and Hamilton and Benson., 2000).

Table 1.1 Amino acids involved in the intermolecular contact of the chimeric and homologous TTR-RBP complexes

The amino acids involved in the interactions from RBP were denoted by E. These residues contacted with those, defined by A, B and C, in the TTR tetramer of the chimeric TTR-RBP complex. D, C and A represented the three monomers of TTR in the homologous complex. cc, the chimeric TTR-RBP complex; hc, the homologous TTR-RBP complex (Monaco, 2000).

RBP	Distance chimeric complex (Å)	Distance homologous complex (Å)	TTR
E-35(Leu)(CD2)	3.41	4.24	B(cc)D(hc)-83(Gly)(CA)
E-67(Trp)(CZ2)	4.20	5.03	C(cc)A(hc)-20(Val)(CG1)
E-67(Trp)(CD2)	3.45	4.04	C(cc)A(hc)-84(Ile)(CD1)
E-89(Lys)(NZ)	2.73	3.57	A(cc)C(hc)-99(Asp)(CO)
E-89(Lys)(NZ)	4.54	4.92	A(cc)C(hc)-99(Asp)(OD2)
E-91(Trp)(NE)	4.07	3.68	A(cc)C(hc)-100(Ser)(CO)
E-95(Ser)(OG)	3.09	2.71	B(cc)D(hc)-114(Tyr)(OH)
E-96(Phe)(CO)	2.69	3.25	B(cc)D(hc)-85(Ser)(N)
E-96(Phe)(N)	3.38	3.46	B(cc)D(hc)-114(Tyr)(OH)
E-96(Phe)(CB)	3.37	3.30	B(cc)D(hc)-84(Ile)(CG2)
E-96(Phe)(CE)	3.44	3.25	C(cc)A(hc)-21(Arg)(CG)
E-97(Leu)(CO)	2.80	3.59	B(cc)D(hc)-85(Ser)(OG)
E-99(Lys)(NZ)	2.70	5.47	A(cc)C(hc)-99(Asp)(OD2)
E-99(Lys)(N)	3.87	4.33	B(cc)D(hc)-85(Ser)(OG)
E-(Retinol)(OH)	3.15	3.48	B(cc)D(hc)-83(Gly)(CO)

2.5.3 Other function of TTR: TTR as a cryptic protease

Up to date, several mutations in TTR gene have been identified and up to 80 of those mutations were revealed related to amyloidosis, which is a generic term that describes several diseases characterized by the deposition of insoluble fibrillar proteins in different organs (for review see Ghiso and Frangione, 2002). The molecular mechanism underlying the aggregation of these soluble proteins is still unclear. However, proteolysis has been proposed to be involved in the process. Recently, a novel function of TTR as a cryptic protease has been demonstrated (Liz *et al*., 2004) and the involvement of the proteolytic ability of TTR to the occurrence of amyloidoses could be summarized in two opposite directions.

One direction, proteolytic activity of TTR has been shown initiating the fibril formation of amyloid proteins. Under physiological conditions, a fraction of 1-2% of TTR in human circulates in high density lipoproteins (HDLs) through binding to apolipoprotein AI (apoA-I). Co-localization of TTR with amyloidogenic apoA-I was first identified in kindred with apoA-I amyloidosis (Sousa *et al*., 2000). Thereafter, TTR has been shown cleaving apoA-I at the C-terminus, and triggering the fibril formation of apoA-I by the cleavage with TTR was proposed (Liz *et al*., 2004). TTR was revealed as a chymotrypsin-like serine protease according to its activity that was inhibited by general serine and chymotrypsin-like serine protease inhibitors. In addition, cleavage by TTR preferred a Phe residue on P1 with the optimum pH of 6.8 (Liz and Sousa, 2005). The protease activity of TTR is not only involved in the amyloid fibril formation that leads to amyloidosis, but also it is important to metabolism of lipid. It was demonstrated that the TTR cleaved apoA-I had decrease of ability to promote cholesterol efflux (Liz *et al*., 2007).

In the oppposite direction, the proteolytic ability leads TTR as sequester in clearing the amyloid proteins or peptides and protects the occurrence of amyloid fibril and, thus, amyloidosis. This role of TTR was revealed with amyloid beta (Aβ) peptide. Aβ is the proteolytic product of amyloid precursor protein (APP), an integral protein that has been cleaved at the C-terminus and yield various length of peptides (Aβ 40-42) (Mori *et al*., 1992; Miller *et al*., 1993 and Rocher *et al*., 1993). These peptides have high potential to aggregate and form amyloid fibril, major participates in the pathogenesis of Alzheimer's disease (AD) (Yamauchi *et al*., 1995).

There are several molecules has been identified as Aβ sequesters to date and one of them is TTR (Schwarzman *et al*., 1994; and Stein *et al*., 2002). The study of Schwarzman and group demonstrated that TTR was the major Aβ binding protein in CSF and the interaction between TTR and Aβ led to a decrease in the Aβ aggregation and, as a consequent, reduced toxicity of the Aβ amyoid (Schwarzman *et al*., 1994 and 1996). The *in vivo* study in the *Caenorhabditis elegans* (*C. elegan*) expressing human Aβ showed that in the presence of TTR, the neurodegeration triggered by the Aβ peptide was reduced (Link, 1995). Moreover, the amino acid residue that involved in the binding interaction between TTR and Aβ were demonstrated situate on surface of the TTR monomer (Schwarzman *et al*., 1996). Since wild type TTR showed the sequestering effect on Aβ, the variation/mutation in amino acid sequence of the TTR monomer have been proposed affects on the sequestration property of TTR. Recently, the *in vitro* studies revealed that different variant TTRs bound Aβ with different strengths and most of them could inhibit aggregation of Aβ peptide. However, there was no correlation between TTR variants and the degree of inhibition/disruption of the Aβ fibrillogenesis (Costa *et al*., 2008).

Very recently, TTR was shown also be able to cleave amidated neuropeptide Y (NPY), and the cleavage of NPY by TTR was demonstrated to be related to functions of TTR in nerves such as promoting the regeneration of neuritis (Liz *et al*., 2009).

3. The evolutionary structure of TTR and the relationship to its functions

3.1 Evolution of primary structure of TTR: changes at the N-terminal region

During evolution of vertebrates, TTR showed a very characteristic changes, which can divide into two groups: the change in tissue pattern of TTR gene expression and those in TTR gene structure. The expression of TTR gene in different tissues especially in liver and choroid plexus during evolution and during development has been studied in many vertebrate species and it found that the expression of TTR gene in choroid plexus is believed to start at the stage of stem reptiles, about 320 million years ago. While, the expression of TTR gene in liver of adult animals appeared relatively late and independently in the vertebrate lineages leading to eutherians, birds and diprotodont marsupials (for review see Schreiber and

Richardson, 1997 and Prapunpoj *et al*., 2000). Moreover, in all eutherians, avian and diprotodont marsupial species, TTR is synthesized in liver during development and in adult, but for fish, amphibians, reptile and Australian polyprotodont marsupials have TTR in their blood only in some stage of development. The hypothesis and update interpretation of these changes was already presented in the review of Richardson (2007). Moreover, Richardson (2007) has been classified the synthesis of TTR in liver and choroids plexus of the brain during evolution and development of vertebrate into three categories including,

1. TTR synthesis only in liver, only during development: fish and amphibians
2. TTR synthesis in the liver during development and in choroids plexus throughout life: reptiles, polyprotodont marsuprials (and possibly monotreme)
3. TTR synthesis in liver and in the choroids plexus throughout life: birds, diprotodont marsuprials, eutherians.

For the primary structure of TTR, there are two type of mutations occurred. The first type is the mutation that distributed randomly along the polypeptide chain of TTR subunit. This leads to the aggregation and intracellular deposition of the aggregated TTR in various organs, leading to amyloidosis. The second is a systemic change that occurred during evolution of vertebrates. Up to date, the amino acid sequence of TTR that has been determined directly or derived indirectly from the cDNA sequence of many vertebrate species including fish (sea bream, *Sparus aurata*: Santos and Power, 1999), amphibians (bullfrog, *Rana catesbeina*: Yamauchi *et al*., 1998; claw African toad, *Xenopus laevis*: Prapunpoj *et al*., 2000b), reptiles (stumpy-tailed lizard, *Tiliqua rugosa*:Achen *et al*., 1993; salt water crocodile, *Crocodylus porosus*: Prapunpoj *et al*., 2002), a bird (chicken, *Gallus gallus*: Duan *et al*., 1991), marsuprials (stripe-faced dunnart, *Sminthopsis macroura*, American short taild gray opossum, *Monodelphis domestica*, sugar glider, *Petaurus breviceps* : Duan *et al*., 1995a; Tammar wallaby, *Macropus eugenii*: Brack *et al.*, 1995; Eastern gray kangaroo, *Macropus giganteus*: Aldred *et al*., 1997) and eutherians (human, *homo sapiens*: Tsuzuki *et al*.,1985; rabbit, *Oryctolagus cuniculus*: Sundelin *et al*., 1985; rat, *Rattus norvegicus*: Dickson *et al*., 1985; Duan *et al*., 1989; Mouse, *Mus musculus*: Wakasuki *et al*., 1985; sheep, *Ovis aries*: Tu *et al*., 1989; pig, *Sus scofa*: Duan *et al*.,

1995b; cow, *Bos Taurus*: Irikura *et al*., 1997; hedgehog, *Erinaceus europaeus*, shew, *Sorex ornatus*: Prapunpoj *et al*., 2000a). Comparison of these amino acid sequences of TTRs revealed high conservation of the sequence during evolution of vertebrates, in particular those residues in the central channel containing binding sites for THs (figure 1.10) (for review see Richardson, 2007). This indicates the important of TTR function on binding to THs. The tremendous changes in TTR subunit during evolution, on the other hand, are concentrated at the first ten amino acids (based on human TTR amino acid sequence) from the N-terminus. This change was from longer and more hydrophobic N-terminal region in the lower vertebrate including avian, reptilian, amphibian and fish to shorter and more hydrophilic in eutherians such as mammal. Three (for birds, reptiles, amphibians and fishes), two (for some marsupials) and five (for *X. laevis*) additional amino acids have been inserted at N-terminus (compared to that of human TTR). Since the N-terminal of TTR subunit is located at the border of exon1 and exon2 of TTR mRNA, therefore, the region of TTR molecule that involved most has been proposed to be intron1.

Analysis and systemic comparisons between nucleotide sequences of TTR cDNAs and genomic DNAs isolated from eutherian, marsupial and birds revealed that the shortening of the TTR polypeptide chain at the N-terminus occurred at the border between exon 1 and exon 2 (Figure 1.11). By comparing the nucleotide sequences of genomic DNA and cDNA at border between exon 1 and exon 2 showed that the position of the 3′ splice site of exon 1 and the 5′ splice site of intron 1 of all studied TTRs remained unchanged (Aldred *et al*., 1997; Prapunpoj *et al*., 2000a, b and 2002, for review see Richardson, 2007). However, all changes occurred in the region of 3′ end of intron 1 and the 5′ end of exon 2. The position of the 3′ splice site of the TTR intron 1 shifted in the 3′ direction in successive steps during evolution of eutherians from their ancestral TTR genes similar to that in fish leading to a successive shortening of the 5′ end of exon 2. The mechanism underlying this splice site shift was revealed occurred by a series of single base mutations that converted codons for amino acids into a new splice recognition site (Aldred *et al*., 1997). According to the fact that the splice site recognition sequence at the 3' end of introns is relatively short, codons for amino acids in particular glutamine, CAA, and histidine, CAC or CAU,

can be converted into the 3' splice site recognition sequence CAG by a single base change i.e. from A, C or U to G. This conversion apparently has occurred at the 5' end of exon 2 of several TTR genes during evolution of eutherians. The histidine codon CAU in marsupials is possibly converted into the consensus recognition sequence CAG by single base change from U to G, and, at least one other single base substitution, G to U or C, have occurred to inactivate the former 3' splice site recognition sequence operating in marsupial. A valine codon in position γ apparently changed into the 3' splice site recognition sequence CAG, by either three base changes or deletion of the valine codon, during the evolution of the TTR gene in marsupial from its ancestor gene similar to that in bird and reptile. In addition, the conversion of the histidine codon CAU (in position ε) or the histidine codon CAC (in position ι) to the consensus sequence CAG occurred during evolution of bird and reptile TTR from the amphibian-like ancestor and that of amphibian from the fish-like ancestor TTR, respectively (Aldred *et al*., 1997; Prapunpoj *et al*., 2000a, b and 2002 and Mazon *et al*., 2007). These changes during evolution of the 5' end of exon 2 of the TTR gene resulted in a progressive movement of the 3' splice site, but not the 5' splice site of intron 1, in the 3' direction and successive steps from fish to amphibian, reptilian and avian, marsupial and, finally, eutherian species, resulting in a progressive shortening of the 5' end of exon 2 and increase in hydrophilicity of the N-terminal region of the TTR subunit. Fore example, N-terminus of chicken TTR is longer and more hydrophobic due to the presence of Val and Leu residues than those of eutherian TTR which have high portion of Glycine and Threonine (Duan *et a*l., 1991 and Aldred *et al*., 1997).

3.2 Changes at the C-terminal region

In addition to the finding of a systemic change of amino acid sequences at the N-terminal region, variation in length and hydropathy of amino acids at the C-terminal region was also observed in some species. The C-terminal regions of TTRs from pig, amphibians and lampreys have more amino acid residues (additional two to three amino acid residues) in comparing to that of human TTR) (Figure 1.10). The C-terminal portion of these animals is relatively more hydrophobic than that in mammalian TTR.

3.3 Effect of the N- and C-terminal regions on function of TTR

Binding affinity of TTR to THs, which could be determined from K_d value, varied among animal species. By using a method developed by Chang *et al*.(1999), the binding affinity of TTR from numerous vertebrate species including eutherians (human, rat and sheep), marsupials (wallabies, wombat and opossums), avians (pigeons, chickens and emus) (Chang *et al*., 1999), reptilians (crocodile) (Prapunpoj *et al*., 2002), amphibian (*X. laevis*) (Prapunpoj *et al*., 2000b) and fish (sea bream) (Santos and Power, 1999 and Yamauchi *et al*., 1999) have been determined. It showed that eutherian TTRs and marsupial TTRs bound T_4 with higher affinity than T_3. While avian, reptilian and amphibian TTRs bound T_3 with higher affinity than T_4**.** Although the piscine TTR bound T_3 with higher affinity than T4 but the specific K_d values has not been elucidated. From the three-dimensional structure of TTR, the N-terminal region protruded from the protein tetramer and was located at the entrance of central channel where the binding sites for THs locate. Taken all together, it was proposed that the N-terminal region of the TTR subunit may affect accessibility of TH to the binding site in the central channel of TTR (Duan *et al*., 1995a).

Many affords have been provided to clearify effects of the N-terminal segment on functions of TTR. The mutation to which amino acid at position 6 of the human TTR subunit has been changed from Gly to Ser (G6S) had higher affinity for T_4 than the wild-type TTR (Fitch *et al*., 1991). Comparative studies of the chimeric TTRs supported this hypothesis (Prapunpoj *et al*., 2002 and 2006). Moreover, by using sea bream TTR as model, length of the N-terminal region of TTR was shown affected on the accession of T_4 to the binding site of TTR (Morgado *et al*., 2008), confirming the possible role of the N-terminal segment on the binding of TTR to TH.

Since C-terminal portion is also located at entrance to the central channel that harbors the binding sites for THs, the effect of this region on the binding affinity of TTR to THs and other ligands should not be excluded. The involvement of the C-terminal regions on functions of TTR those have been studied so far include the effect on binding to a specific retinol plasma transporter, RBP (Naylor and Newcomer, 1999) and that on the pathogenesis of amyloidosis (Solovyov *et al*., 2006).

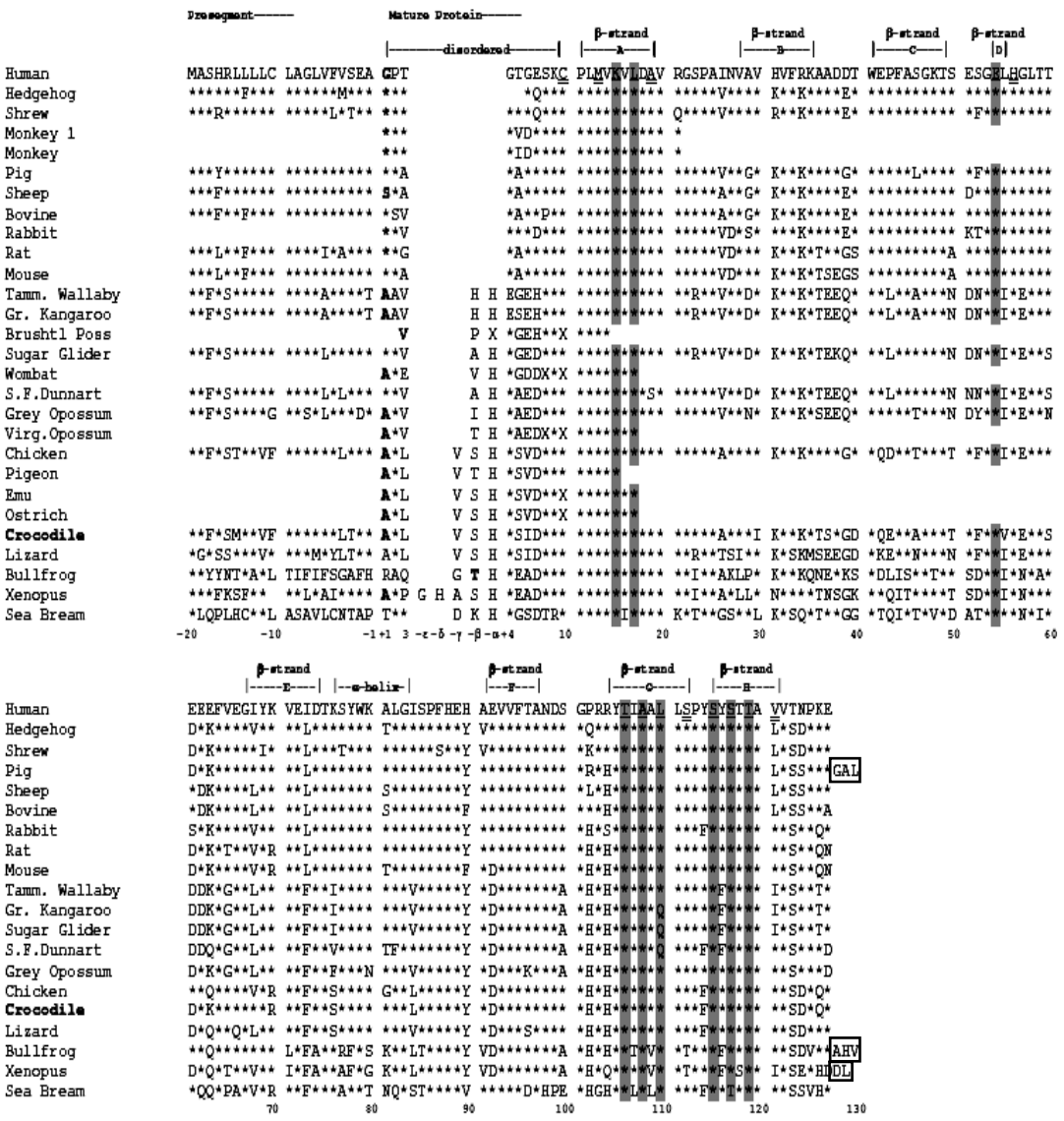
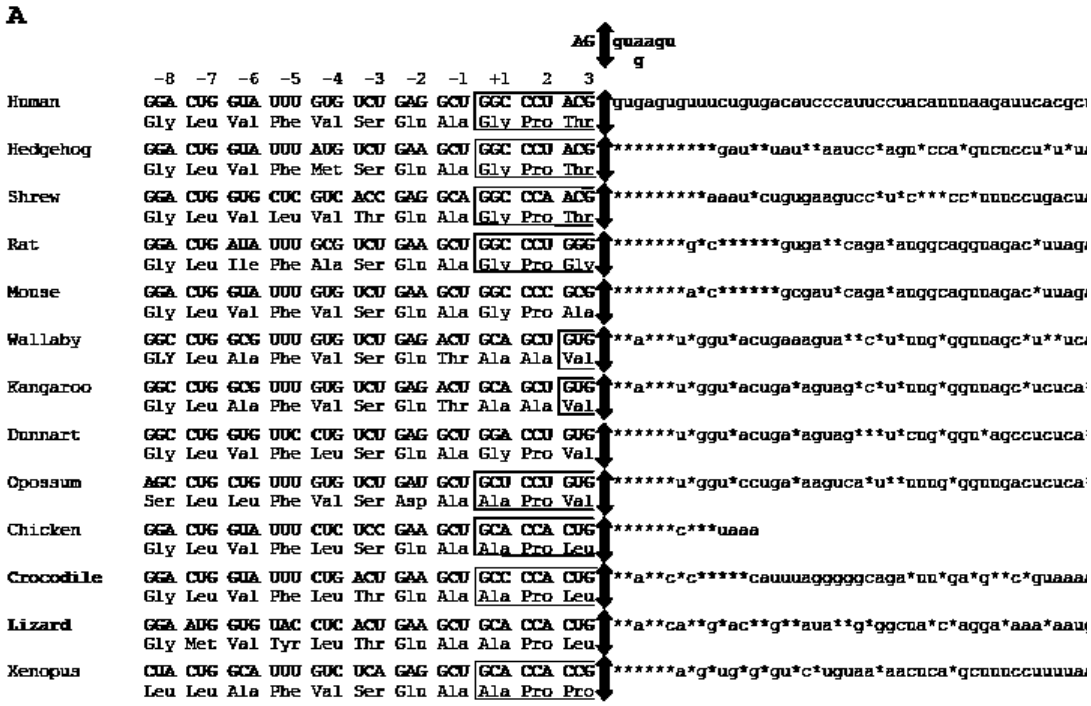

Prosegment
Mature Protein
disordered
β-strand A
β-strand B
β-strand C
β-strand D
β-strand E
α-helix
β-strand F
β-strand G
β-strand H
Human
Hedgehog
Shrew
Monkey 1
Monkey
Pig
Sheep
Bovine
Rabbit
Rat
Mouse
Tamm. Wallaby
Gr. Kangaroo
Brushtl Poss
Sugar Glider
Wombat
S.F.Dunnart
Grey Opossum
Virg.Opossum
Chicken
Pigeon
Emu
Ostrich
Crocodile
Lizard
Bullfrog
Xenopus
Sea Bream

Figure 1.10 Comparative alignment of structure of vertebrate TTRs

Amino acid sequences or that derived from the cDNA sequences of vertebrate TTRs are aligned with the amino acid sequences of human TTR. Single-letter amino acid abbreviations are used. X indicates an amino acid could not be unambiguously identified by Edman degradation. Asterisks are residues identical to those in human TTR. Gaps were introduced for proper alignment. Features of secondary structure of human TTR are indicated above the sequences. Numbering of residues is based on that for human TTR. Negative numbers indicate the residues in the presegment; positive numbers represent residues of the mature protein. Negative Greek letters, α, β, δ, γ, and ε were introduced to indicate positions of residues in non-eutherian species. Bold letters indicate the first amino acid of mature TTRs. Double underlining indicates amino acid residues located in the central channel. Gray shading indicates amino acid residues, in the central channel, that are involved in binding thyroid hormones (from Prapunpoj *et al*., 2002). Extra amino acid residues found in TTRs from pig, bullfrog and *X. laevis* are in boxes.


A
AG guaagu
g
−8 −7 −6 −5 −4 −3 −2 −1 +1 2 3
Human
Hedgehog
Shrew
Rat
Mouse
Wallaby
Kangaroo
Dunnart
Opossum
Chicken
Crocodile
Lizard
Xenopus

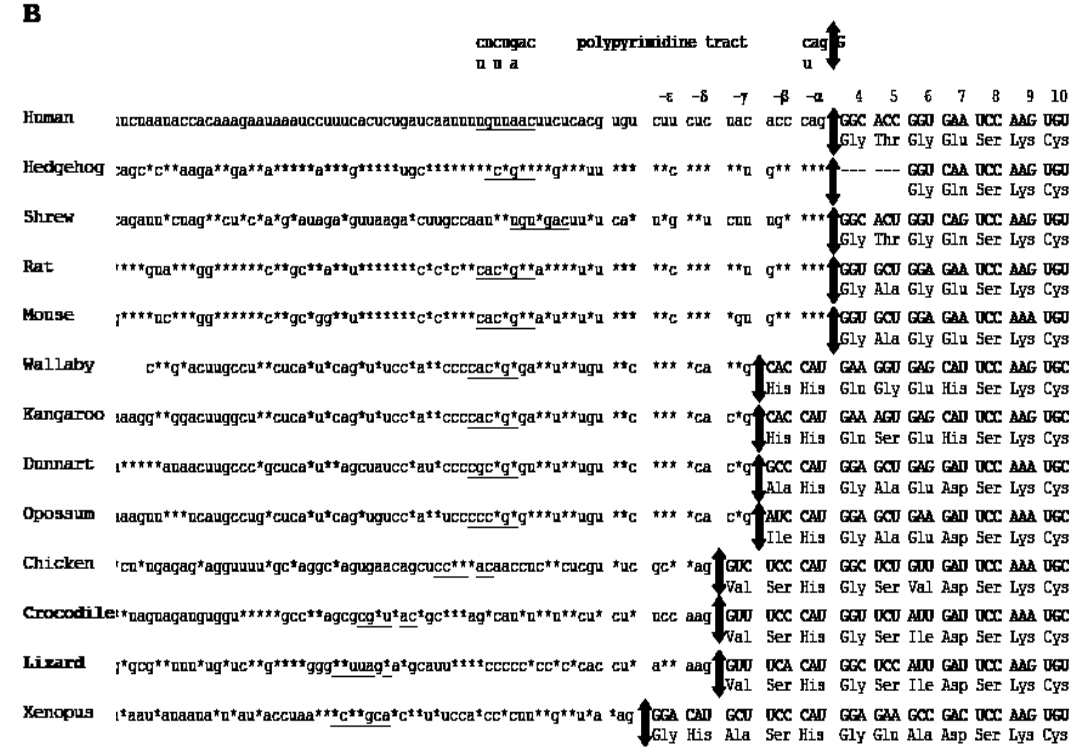

B
cacugac
u u a
polypyrimidine tract
cag G
u
−ε −δ −γ −β −α 4 5 6 7 8 9 10
Human
Hedgehog
Shrew
Rat
Mouse
Wallaby
Kangaroo
Dunnart
Opossum
Chicken
Crocodile
Lizard
Xenopus

Figure 1.11 Comparison of the nucleotide and derived amino acid sequences of TTR precursor at the exon 1/intron 1 (A) and intron 1/exon 2 (B) borders

The nucleotide sequences in exons are in bold upper case, while those in introns are in lower case. The deduced amino acid sequences are given beneath the nucleotide sequences. The consensus recognition sequences for splicing are indicated in bold above the positions of the splice sites in human TTR mRNA. The amino acid sequence of the N-terminus, determined by Edman degradation of the mature or the recombinant TTRs, are indicated by a box open at the right end.

(Modified from Prapunpoj *et al*., 2002; for more details see Prapunpoj *et al*., 2002).

4. TTR-like proteins (TLPs) in non-vertebrate species

The presence of TTR-like proteins or TLPs has appeared in databases since the late 1990s. These TLPs have been identified in bacteria, plants and animals from non-vertebrates to vertebrate species (Eneqvist *et al*., 2003 and Hennebry *et al*., 2006a). It has been demonstrated that these TLP genes are widely distributed in nature whereas TTR gene is found only in vertebrates. The conservation of this gene during evolution might be pointed to its important in all organisms. Five open reading frames (ORFs) that could potentially encode TLPs were studied in two *C. elegan*, *Salmonella Dublin*, *E. coli* and *Schizosaccharomyces pombe* (for review see Richardson 2007). The three-dimensional study of TLP from *E. coli* revealed the TLP structure similarity to TTR from vertebrates. Moreover, the recombinant TLP from this organism together with that from bacterium *S. Dublin*, an invertebrate animal *C. elegan* and plant (*Arabidopsis Thaliana*) was found to be tetrameric, though TLPs from *E. coli* and *S. Dublin* could not bind to THs (Eneqvist *et al*., 2003 and Hennebry *et al*., 2006a). The possible role of these TLPs is still under investigated but the RNAi study in *C. elegan*, suggested the involvement of this protein in uricase metabolism (Eneqvist *et al*., 2003).

The first X-ray crystal structure of *S. Dublin* was studied by Hennebry *et al*. (2006b). TLP was shown having an overall three-dimensional structure similar to that of vertebrate TTRs, despite low sequence identity. The structural difference within the monomer was restricted to the flexible loop regions, while the sequence variation at the dimer-dimer interface resulted in a central channel with characteristic different from those of TTR. The differences in amino acid composition and configure of this central pocket led to decrease the capability of TH to bind in the TLP channel (Hennebry *et al*., 2006b; for review see Richardson 2007). The finding that TLPs have the similar three-dimensional structure to those of TTRs but have different sub-cellular localization (change from cytosolic, peroxisomal, or periplasmic in some microbe to extra cellular in vertebrates) and function (changed from function as 5-HIUase in some bacteria to THDP in vertebrates) during evolution has been suggested making these proteins as a good model for study of the relationship of evolution and protein function which should leading to more understanding about selection pressure

to maintain structure which in turn implies to importance of function even though the function has changed (Richardson, 2007).

5. Recombinant protein production

Recombinant proteins have been widely used nowadays for many applications from *in vivo* and *in vitro* functional studies to large scale synthesis for therapeutic used. The development in the DNA technology make it is possible to produce recombinant proteins by using a variety of expression system including bacterial, insect, mammalian and yeast systems.

Among the systems that available for heterologous protein expression, the most attractive and commonly used is the expression system of bacteria in particular *E .coli*. This gram negative bacterium has become an organism of choice for many reasons. It is a single cell organism that has an ability to produce protein in large amount in a short time period since it can grow very fast in comparison to other cell types such as mammalian cells. It does not require special media and much more facilities for growth and maintenance so that the production cost is very cheap when compared to the other expression system. In addition, the ways to transform *E. coli* with foreign DNA is easy and requires small amount of DNA. Although the simplicity of *E. coli* makes it easy to work with, it also has limitation. The drawback of this expression system is that *E. coli* is a prokaryote which doesn't has ability to glycosylate the proteins it produced. For example, although *Neisseria meningirulls* have been demonstrated to have ability to process O-glycosylation to some of its endogenous proteins, the O-linked trisaccharides added are different from the O-linked sugars found in eukaryote (Marston and Hartley, 1990). In addition, *E. coli* usually synthesize the recombinant proteins in an insoluble and inactive form in cytoplasm that need additional step for recovery of the active protein.

Since, *E. coli* has a limitation to produce eukaryotic products. Other expression systems include those from mammalian, insect and yeast cell are required. Among these, yeast cell is the best choice. The advantages of yeast over other expression systems are that yeast combine good characteristics of both microorganism and eukaryote. As a microorganism, it can grow very fast but at the meantime it has a sub-cellular machinery to perform the post translational modification process as found in

eukaryotes (Cregg *et al*., 1993). The other advantages of yeast cell are that it has advance protein folding pathway for heterologus proteins and it can secrete correctly folded and processed protein into culture medium. The other advantages of yeast over bacteria are that proteins that accumulated as insoluble inclusion body in *E. coli* are often soluble when expressed in yeast (Ridder *et al*., 1995b). Moreover, a problem of heterologous proteins degradation that usually found when using *E. coli* can be reduced by using yeast. In comparison to mammalian cells, yeast can rapidly grow and require simple media for growth.

Another alternative system is insect cell. This expression system becomes a popular method because of many reasons. The insect cells are higher eukaryote than yeast and they have ability to perform most of the post-translational modifications which exist in higher eukaryotes including glycosylation (Bei *et al*., 1995), phosphorylation (Miyamato *et al*., 1985; Jeang *et al*., 1987 and Nyunoya *et al*., 1988), acylation and palmitylation (Lanford, 1988). Moreover, this system has been reported produces high level of the desire proteins while retains its functional activity (Verma *et al*., 1998). Two common methods have been adopted to introduce DNA of interest into insect cell, the baculovirus-mediated transformation and the stable transformation of the gene under the control of suitable promoter. The former system is the most popular for the insect cell expression system as large amounts of active protein could be produced. Moreover, higher expression level (1 to 500 mg of recombinant protein per liter of infected cells) and a highly restricted host range of the baculovirus make insect cells are safer and more attractive than mammalian cells (Groner, 1986; Luckow and Summers, 1988). However, many factors have to be concerned in order to get succeed in expression of a foreign gene. These include the requirement for a very good quality media, an elaborated facilities for growth and maintenance since this cell types is very sensitive to stress and need high level of oxygen for viable. Although post translational alterations of the synthesized proteins take place in the insect cells, some mechanism processes are slightly different from that occurs to some eukaryotic proteins. The other limitation of baculovirus system is that the protein expression is controlled by a very late viral promoter and the highest of expression occurs only when cell die from the viral infection. From this drawback, stable transformation under the control of appropriate promoter is required. There are many

host cells recommend for this transformation, normally are derived from dipterans insect such as fruit fly and mosquito such as the Schneider2 (Schneider, 1972 and Sang 1981) and C7 from *Aedes Albopictus* (Sarver and Stollar, 1977). The common promoters used are the drosophila metallothionine (Verma *et al*., 1998) and the baculovirus immediate early (ie) promoters from *Bonbyx mori* multicapsid nuclear polyhedrosis virus (MNPV) (for review see Pfeifer, 1998).

The other expression system that has been widely used is mammalian cells. The production of proteins by mammalian cells is an important tool in numerous scientific and commercial areas including in the life science researches, for the therapeutic usages, for the protein engineering and in the cell-based biosensors (www.genwaybio.com, 4/02/09). The main advantages of this gene expression system is that the signals for synthesis, processing and secretion of the eukaryotic proteins are properly and efficiently recognized by the mammalian cells (even though there are differences between species), so the proteins those are produced have the structural and functional features similar to those in native. There are two general methods for introduction of the foreign DNAs into the mammalian cells: transfection and infection. For transfection, the foreign DNA will be directly transferred into cells by chemical or physical methods. Whereas the infection, the foreign DNA will be introduced into cell via an infection by virus that carried the DNA of interest. Although this expression system has been extensively used, in particular for the therapeutic applications, there are some limitations i.e. the mammalian cell expression system is highly cost, require complicate steps of the process and need very well take care culturing.

Even though there are several expression systems offer for the recombinant protein production nowadays, each system has its advantages and limitations. The choice of expression system depends on many factors, for example, the nature of protein molecule, the experience of a laboratory to use different expression system, the quantity and quality of recombinant protein needed, the need for posttranslational modifications and processing as well as production costs (for review see Verma *et al*., 1998). For the recombinant TTR, the synthesis in large amount has been reported using two expression system the *E. coli* and a methylophilic yeast, *Pichia pastoris*.

6. Heterologous protein expression in *P. pastoris*

Heterologous protein expression in yeast has been developed to be used as a replacement of bacterial cells. There are two strains of yeast that have been extensively used, *Saccharomysis cerevisiae* and *P. Pastoris*. *S. cerevisiae* was the first eukaryotic expression system that was developed. This yeast specie is popular due to large amount of its genetic information that make scientists are familiar with. However, the large scale synthesis of the recombinant proteins in *S. cerevisiae* is not optimal due to some problems. The expression system of *P. pastoris*, therefore, has been later developed and used as an alternative system (Zhang *et al*., 2000).

P. pastoris has many advantages of eukaryote expression. Their growth could be raised up to a very high density in a simple defined medium. *P. pastoris* has a strong inducible promoter that can be used for protein production and is capable to generate post translational modifications, which are similar to those of higher eukaryotes. In addition, there are commercially available methods, host strains and expression vector for genetic manipulations (Invitrogen Corporation, Carlsbad, CA, USA) (Cregg *et al*., 1993; Higgin and Cregg, 1998b; for review see Zhang *et al*., 2000). Over 100 foreign proteins from bacteria, fungi, plants, invertebrates and vertebrates including human have been expressed in *P. pastoris*. This heterologous gene expression system become a good choice for NMR analysis of the protein that can not be refolded after isolation from inclusion bodies or required post translation alterations for properly fold of function (Wood and Komives, 1999).

The promoter for alcohol oxidase gene (*AOX*) is used as a strong inducible promoter in the expression system of *P. pastoris*. This promoter has a related function to the methylotrophic nature (or methanol utilization) of the yeast (Veenhuis *et al*., 1983). In the first step of methanol utilization is an oxidation of methanol to formaldehyde and hydrogen peroxide which is catalyzed by enzyme alcohol oxidase (AOX) in peroxisome. The hydrogen peroxide that is generated will then be degraded to oxygen and water by enzyme catalase, the classic peroxisomal marker enzyme. Since AOX has poor affinity to oxygen, yeast need to synthesize for a large amount of the enzyme to overcome the low specific activity of the enzyme (Van Dijken *et al*., 1976; Couderc and Barratti, 1980; Veenhuis et al., 1983 and Giuseppin *et al*., 1988). The genome of yeast contains two copies of AOX gene, *AOX1* and *AOX2*. The *AOX1*

promoter regulates 85% of the AOX activity in the cell and is the promoter that has been used to drive heterologous protein expression in *P. pastoris* (Cregg *et al*., 1989). The expression of *AOX1* gene is tightly regulated. It is induced by methanol and the control is largely at the transcriptional step (Cregg *et al*., 1985; Ellis *et al*., 1985 and Cregg *et al*., 1989). The regulation of *AOX1* gene appear to involve with two mechanisms: the repression/de-repression mechanism and the induction mechanism. The promoter is suppressed during growth of yeast in many common carbon sources, i.e. glucose, glycerol and ethanol, but it is induced during growth by methanol (Tschopp *et al*., 1987b).

AOX1 promoter has been widely used for heterologous protein expression. Most genes of interest are placed under control of this promoter. The *Pichia* transformants will be growth to generate biomass on a repressing carbon source and then production of foreign protein initiated by shifting to a medium containing methanol as the sole carbon source. Since methanol is an inducer for *AOX1* promoter when this promoter is induced, the foreign protein will be co-expressed. Besides using *AOX1* as a promoter in driving the gene expression, the other alternative promoters have been recommended including *GAP*, *FLD1*, *PEX8* and *YPT1* promoters (Cereghino and Cregg, 2000).

Two strategies have been used to integrate vector into the *P. pastoris* genome, gene insertion and gene replacement (Cregg *et al*., 1985). All *P. pastoris* expression strains are derived from NRRL-Y 11430 (Northern Religional Research Laboratories, Peoria, IL) (for review see Higgind and Cregg, 1998b). There are three phenotypes of *P. pastoris* expression strains regarding to methanol utilizing ability, this include methanol utilization positive (Mut^+), methanol utilization slow (Mut^s) and methanol utilization negative (Mut^-) (for review see Higgind and Cregg, 1998b).The most commonly used expression host is GS115 (*his4*). This strain contains the *AOX1* and *AOX2* genes and grows on methanol at wild type rate (Mut^+) because its growth on methanol mainly relies on the presence of the *AOX1* gene. The other commonly used as a host is the protease deficient strains, in particular SMA1168, which has been showed to be effective in reducing degradation of some foreign proteins (White *et al*., 1995 and Brierley, 1998). However, the limitation of this strain is the slow rate of

growth and difficulty of transformation that, as a consequence, brings to lower transformation efficiency.

P. pastoris has a potential to produce foreign proteins either intracellularly or extracellularly. Since this yeast grow on simple mineral media and secrete only low levels of endogenous proteins, the heterologous protein that has been secreted constitutes the vast majority of total protein in the medium. This makes it easier to purify from other proteins in the culture medium. Several different signal recognition sequences have been successfully used. These include the native signal sequences of the heterologous proteins, the signal sequence of α-factor prepropeptide from *S. cerevisiae* and the sequence derived from the acid phosphatase gene (*PHO1*) of *P. pastoris*. Among these, the α-factor prepropeptide has been used with the most succeed (for review see Cereghino and Cregg, 2000).

The eukaryotic posttranslational events such as signal sequence processing, protein folding, disulfide bridge formation and glycosylation take place during the passage of protein through the secretary pathway. Post translational modifications often involved the addition of oligosaccharides to 0- and N-asparagine-linked sites on proteins. Types of sugar in the oligosaccharides including size and structure of these sugar polymers can affect the folding ability of a protein and, as a consequence, its activity and immunogenicity. *P pastoris* has an ability to add both 0- and N-linked carbohydrate moieties to the secreted proteins (Goochee *et al*., 1991). However, the O-link glycosylation of *P. pastoris* is different from that of mammals. In mammals, the O-linked oligosaccharides are composed of variety of sugars, whereas only mannose (Man) residues are added to the O-linked of protein produced in *P. pastoris*. In addition, different host may add O-linked sugars on different residues in the same protein, therefore, additional mannose to foreign protein can occur even if that protein is not glycosylated by its native host (for review see Cereghino and Cregg, 2000). For the N-linked glycosylation, several foreign glycoproteins secreted by *P. pastoris* have a typical eukaryotic glycosylation pattern, which is contrast to those obtained from *S. cerevisiae* (Montesino *et al*., 1998). The secreted proteins from *S. cerevisiae* and *P. pastoris* showed difference in the N-linked oligosaccharides structure though the majority of N-linked oligosaccharide in these two yeasts are rich in mannose. Length of the oligosaccharide is much shorter in *P. pastoris* than in *S. cerevisiae* (Grinna and

Tschopp, 1989). However, the long oligosaccharide is one of important feature of hyperglycosylation of the proteins produced by *S. cerevisiae*. Only few examples of hyperglycosylation of foreign proteins were reported from using the expression system of *P. pastoris*. Another difference between glycosylation by these two yeast species is that glycans synthesized by *P. pastoris* do not have α-1,3 linkage mannose, which, in contrast, is a typical feature of *S. cerevisiae* (Cregg *et al*., 1993). Since the α-1,3 linkage of glycoprotein produced by *S. cerevisiae* are involved to the highly antigenic nature of the proteins, this make it unsuitable for human pharmaceutical uses (Cregg *et al*., 1993). The recombinant proteins produced by *P. pastoris*, in comparison to *S. cerevisiae*, more desirable for use in human.

7. Heterologus expression of TTR

The synthesis of recombinant TTR has been attempted on TTR from many species with the main purpose for analysis on the molecular basis of genetic disorders and mechanism of amyloid fibril formation (Furuya *et al*., 1989 and Murrel *et a*l., 1992; Rosen *et al*., 1994 and Liz *et al*., 2004), also for study of structural relationship to functions of TTR (Prapunpoj *et al*., 2000a, 2002 and 2006 and Morgado *et al*., 2008). Large scale synthesis has been reported using two expression systems the *E. coli* and the yeast *P. Pastoris*.

By using the *E. coli* expression system, the synthesis of the recombinant human TTR, both wild-type and variants, have been successfully synthesized with variety of expression vectors. In using pQE30 (Matsubara *et al*., 2003), TTR was expressed under the control of a lipoprotein promoter. Several TTR variants including Gly6Ser, Leu55His, Thr60Ala, Ile84Ser and Ala109Thr were produced using pCZ11 (Murrel *et al*., 1992). All the recombinant TTRs produced by using the expression system of *E. coli* were reported forming tetramer with similar size to the native TTR, and these proteins retained ability to bind with T_4 (Furuya *et al*., 1989; Murrel *et al*., 1992 and Mutsubara *et al*., 2003). In addition, the recombinant variant TTRs still had ability to form amyloid fibril at acidic pH similar to the TTR isolated from human plasma (Mutsubara *et al*., 2003).

For the production of recombinant TTRs by using *P. pastoris*, few types of TTRs and chimeric TTRs have been successfully produced so far. These include the native

TTRs from *X. laevis* (Prapunpoj *et al*., 2000a) and *C. porosus*, and a chimeric *C. porosus* TTR (Prapunpoj *et al*., 2002). All of the recombinant TTRs produced by *P. pastoris* had similar physiochemical properties including subunit masses, molecular weight of tetramer, binding to THs and RBP similar to those reported for the TTRs in nature.

Objectives

To elucidate influence of N or C-terminal region on functions of TTR by

1. Synthesize the recombinant wild type TTRs and the recombinant chimeric TTRs, which N- or C-terminal sequence was changed, by using the heterologous protein synthesis system of *P. pastoris*.

2. Purify and determine general physicochemical properties of the recombinant TTRs.

3. Determine the binding affinities to THs and RBP, and proteolytic property of the recombinant TTRs.

CHAPTER 2

MATERIALS AND METHODS

1 Materials

1.1 Instruments

Instruments	Model	Company
Autoclave	ES-315	Tomy
Balance (4 digits)	AB204-S	Mettler
Balance (2 digits)	PG5002-S	Mettler
Centrifuge	Anti J-30 I	Beckman
Centrifuge	J2-21	Beckman
Centrifuge	5804R	Eppendorf
Centrifuge	Harrier 18/8 (MSE)	SANYO
Fraction collector	2110	Bio-Rad
Gel Document (Lab Works 4.0)	C-80	UVP
Horizontal Electrophoresis	B1	Owl Scientific
High performance liquid chromato-Graphy (HPLC)		Waters
Incubator		Memmert
Microcentrifuge	260D	DENVILLE
Micropipette		Gilson, Labnet, Labmate, Nichipet EX
Oven	240 litre	Binder
Orbital shaking incubator		GallenKamp SANYO
Orbital shaker	SH 30	FINEPCR
Orbital shaker	MS-OR	Major Science
pH meter	713	Metrohm

Instrument	Model	Company
Power supply	ELITE 300 plus	Wealtec
Power supply	PowerPac 3000	Bio-Rad
Preparative gel electrophoresis	PrepCell 491	Bio-Rad
Slab gel electrophoresis	AE-6450	ATTO
	Mini Protean 3 cell	Bio-Rad
	Mini Protean-II	Bio-Rad
Spectrofluorometer	RF-1501	Shimadzu
Spectrophotometer	8453	Hewlett-Packard
Spectrophotometer	G20	Thermo
Stirrer		Corning
Vortex-mixer	VX 100	Labnet
Water Bath	WB-710M	Optima

1.2 Chemicals

1.2.1 Analytical grade

Chemical	Company
Absolute ethanol	Normapur
Acetic acid	Lab Scan
Acrylamide	Fluka
Agar	Merck
Argento nitrate (Silver Nitrate)	Merck
Biotin	Fluka
Bis-acrylamide	Fluka
Boric acid	Merck
Bovine serum albumin	Sigma
Calcium chloride	Merck
Coomassie brilliant blue G-250	Bio-Rad
Coomassie brilliant blue R-250	Bio-Rad

Chemical	Company
D-glucose	Univar
Dimethylsulfoxide	Lab Scan
Di-potassium hydrogen phosphate	J.T. Baker
Dithiothreitol	Bio-Rad
Ethylene diamine tetraacetic acid (EDTA)	Carlo
Glycerol	Univar
Glycine	Fisher
Methanol	Lab Scan
Peptone	Merck
Potassium dihydrogen phosphate	Fisher
Silver nitrate	Merck
Sodium carbonate	Merck
Sodium chloride	Lab Scan
Sodium dodecyl sulfate (SDS)	Finechem
Sorbitol	Sigma
Tris (Hydoxymethyl)- methylamine	USB
Tryptone	Merck
Tricine	USB
Yeast extract	Merck
Yeast nitrogen base	Difco

1.2.2 Molecular biology grade

Chemical	Company
Agarose	GenePure
Ampicillin	Calbiochem
100 bp DNA ladder	New England Biolabs (NEB)
*Bam*HI	NEB
*Eco*RI	NEB

Chemical	Company
Ethidium bromide	Promega
pGEM-T Easy vector	Promega
Ethidium bromide	Promega
pGEM-T Easy vector	Promega
pDrive vector	Invitrogen
*Xho*I	NEB

1.2.3 Reagent kits

Reagent	Company
QIA kit for plasmid purification	QIAGEN
QIA kit for PCR purification	QIAGEN
QIA kit for gel extraction	QIAGEN

1.3 Yeast and Bacterial cells

P. pastoris strain GS115 and *E. coli* DH5α are gifts from Professor Schreiber, University of Melbourne, Australia. *P. pastoris* strain SMD 1168 is a product of Invitrogen. *E. coli* strain JM 109 are products of Promega.

2. Methods

2.1 Preparation of the recombinant *Pichia* clones for wild type and chimeric human TTRs and chimeric crocodile TTRs

2.1.1 Construction of wild type and chimeric human TTR and chimeric crocodile TTR cDNAs

The cDNAs coding for a wild type human TTR, a chimeric human TTR, and three chimeric crocodile TTRs were constructed in this thesis.

The human TTR cDNAs with compatible restriction ends for ligation into pPIC 3.5 (*Bam*HI at 5′ end and *Eco*RI at 3′ end) and pPIC9 (*Xho*I at 5′ end and *Eco*RI at 3′ end) expression vectors were generated and amplified by PCR. The

amplification was performed as previous described (Prapunpoj *et al*., 2002). The reaction mixture (100 μl) contained 50 ng of human wild type TTR cDNA and 40 pmol of the each appropriate primers (Table 2.1) was carried out in a thermal cycler (Eppendorf) with an initial denaturation step at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 30s, annealing at 53 °C for 30s and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. After amplification, the PCR product was analyzed on 1% agarose gel containing 0.4 μg/ml ethidium bromide. The reaction mixture containing a single amplified human TTR cDNA band with an expected size was further purified using the PCR purification kit (QIAGEN). Then, the purified TTR cDNA was ligated into pGEM T easy (Promega) for *in vivo* amplification in bacterial cell. Ligation reaction mixture (10 μl containing 20 ng of the DNA, 50 ng of pGEM Teasy vector) was set up and the reaction was carried out in the presence of T4 DNA ligase (3 Weiss units) at 4 °C for 16-18 h.

Three types of chimeric crocodile TTR cDNAs, i.e. the hu/croc TTR cDNA that would code for residue Gly1 to Glu7 of human TTR and residues Ser8 to Glu127 of crocodile TTR, the truncated croc TTR cDNA that would code for residue Ser8 to Glu127 of crocodile TTR, and the pigC/croc TTR cDNA that would code for residue Ala1 to Ala120 of crocodile TTR and residue Leu121 to Leu130 of pigTTR were amplified by PCR using *C. porosus* TTR cDNA as a template. While one type of the chimeric human TTR cDNA, i.e. the croc/hu TTR cDNA that would code for residues Ala1 to Asp7 crocodile TTR and residue Ser8 to Glu127 of human TTR was amplified using human TTR cDNA as a template. The specific primer pairs (Table2.1) were incorporated into the reaction mixture to alter the nucleotide sequences of the original cDNA templates to those of the chimeric TTRs and generate the restriction sites for *Xho*I at 5′ end and *Eco* RI at 3′ end of the chimeric TTR cDNAs for ligation into pPIC9 expression vector. The amplification by PCR of all chimeric TTR cDNAs was carried out with the same condition as that described for the recombinant human TTR cDNA, except the annealing temperature at 56°C and 60^{o}C were used in the amplification for pigC/croc TTR cDNA (Table 2.1). The PCR products were analyzed, purified and ligated into pGEM Teasy or pDrive vector as described for human TTR cDNA.

2.1.2 Preparation and *in vivo* amplification of the recombinant TTR vectors for expression in *P. pastoris*

The TTR cDNAs ligated with pGEM Teasy or pDrive vector was transformed into the competent *E. coli* DH5α, prepared by $CaCl_2$ method as described in section 2.11**.** Then, the *E. coli* was plated onto LB agar containing 100 μg/ml ampicillin. After incubation for 14-16 h, three to five single white colonies were grown in 2 ml of Luria-Bertani (LB) medium containing 100 μg/ml ampicillin at 37°C, overnight. The plasmid was purified using the plasmid purification kit (QIAGEN), followed by double digestion with *Bam*HI and *Eco*RI or with *Xho*I and *Eco* RI for 2 h prior the cDNA insert was purified by QIAquick Gel Extraction kit (QIAGEN) as described in 2.12 and further ligated into the *Pichia* expression vector pPIC3.5, which was previously digested with *Bam*HI and *Eco*RI, or pPIC9, which was previously digested with *Xho*I and *Eco*RI. The ligation was carried out with T4 DNA ligase at 4°C overnight, and the recombinant expression plasmid was *in vivo* amplified by introduction the plasmid into *E. coli*. The DNA plasmid was isolated, purified and checked size by restriction analysis. The insertion direction of the DNA in the vector was determined by DNA sequencing, using 5′*AOX1* primer (5′-GACTGGTTCCAATTGACAAGC-3′). Finally, the recombinant plasmid was linearlized by digestion with *Sal*I at 37°C for 2 h and purified by phenol/chloroform extraction. The DNA was precipitated with ethanol and dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer) prior to being transformed into *Pichia* cells.

2.1.3 Transformation of *P. pastoris*

The TTR cDNA plasmid was introduced into the competent *Pichia* strain GS115 or SMD1168 by an electroporation using Gene Pulser (Bio-Rad) and followed the protocol recommend by the company. In brief, 80 μl of the competent *Pichia* cells were mixed with ~1.2 μg of the linearized plasmid, then the cell mixture was transferred to an ice-cold 0.2 cm electroporation cuvette. Cells were incubated on ice for 5-10 min and electroporation was carried out at 1.5 kV, 25 μF and 400 Ω, generating pulse lengths of ~7.68 milliseconds with a field strength ~1.37 kV/cm. Then, the cell suspension was immediately flushed with 1 ml of ice-cold 1 M sorbitol,

and all of the mixture was spread onto the minimal dextrose medium (MD) agar plate and cells were allowed to grow at 30^{o}C for 3 days.

2.1.4 Screening for His^{+}Mut^{+} transformant

Pichia transformants with phenotype His^{+}Mut^{+} (histidine synthesis and methanol utilization plus) were identified and selected form His^{+}Mut^{s} (histidine synthesis and methanol utilization slow) by growing the transformants on two selective agar media, minimal dextrose medium (MD) and minimal methanol medium (MM). A single colony of His^{+} transformant from an original MD plate (section 2.1.3) was picked with a sterile toothpick and patched onto MM and, subsequently, onto MD plate. Plates were incubated at 30°C for 2 to 3 days. Growth of the His^{+}Mut^{+}, but not His^{+}Mut^{s}, is similar on both MM and MD. The screening for His^{+}Mut^{+} was performed from 80 to 100 colonies of the His^{+} transformants, and up to 30 single colonies of the His^{+}Mut^{+} transformants were selected for synthesis of the recombinant protein.

2.2 Synthesis of recombinant TTR

2.2.1 Small scale synthesis

The synthesis of the recombinant TTRs from *Pichia* transformants were first carried out in a small scale. A single colony of a *Pichia* clone on yeast extract peptone dextrose (YPD) plate was inoculated into 5 ml of the buffered medium containing glycerol (BMGY). Cells were grown at 30°C in an orbital-shaking incubator (200 rpm) until OD_{600} reaches 2 to 6 (~18 h). Then, cells were collected by centrifugation at 2,500 rpm for 5 min at room temperature and transferred to 5 ml of the buffered medium containing methanol (BMMY). Cells were grown to an OD_{600} ~1.0. Methanol was added every 24 h to maintain the level of methanol in the culture to 0.5% for 3 to 5 days. After induction, the culture supernatant was collected by centrifugation at 2,500 rpm for 5 min and kept at -20°C until used. The secreted proteins in the yeast culture supernatant were analyzed by SDS-PAGE and/or native-PAGE and the protein bands were detected by silver staining.

2.2.2 Large scale synthesis

In order to obtain large or sufficient amount of the recombinant protein for further study, a large scale preparation of the recombinant TTR was performed by using the shaking flask culture method using the same culturing condition as that for

the small scale synthesis. A single colony of the *Pichia* clone from YPD plate was inoculated into 10 ml of BMGY and the yeast cell was grown at 30°C for 16-18 h. Thereafter, 5 to 10 ml of the overnight culture was transferred into 300 ml of BMGY in a 1-liter flask and cells were allowed to grown until OD_{600} reached 2 to 6. Then, cells were collected by centrifugation at 2,500 rpm for 5 min at room temperature, suspended in 300 ml of BMMY in a 2-liter flask to a density equivalent to OD_{600} 1.0. Cells were grown at 28^{o}C and induction for synthesis with methanol was performed for 3-5 days. The concentration of methanol in the culture was maintained to 0.5 to 1% through the induction period. Thereafter, the culture supernatant was collected by centrifugation at 2,500 rpm for 10 min at 4^{o}C. The levels of synthesis and secretion into culture medium of the recombinant TTRs were determined by native-PAGE and SDS-PAGE followed by silver staining.

2.3 Purification of the recombinant TTR

The recombinant TTRs were purified from the *Pichia* culture supernatant by preparative native-PAGE using Prep Cell 491 (Bio-Rad), and sometimes followed by affinity chromatography using a human retinol binding protein (hRBP) couple to Sepharose (NHS-activated Sepharose, Hitrap NHS-activated HP, GE Healthcare) using the method described by Larsson *et al*. (1985) .

Purification by the preparative native-PAGE was carried out using the condition recommended in the instruction manual of the company. The *Pichia* culture supernatant was concentrated by ultrafiltration prior applying onto a polyacrylamide gel tube (12% and 4% for resolving and stacking gels, respectively). The protein separation was performed at constant power (17 Watts), and protein bands were eluted out with 50 mM Tris-HCl, pH 7.4 at a constant flow rate of 1 ml/min. The presence of TTR in the eluted fractions was analyzed by native-PAGE followed by silver staining. The fractions containing TTR were pooled, concentrated and stored at -20^{o}C until further analysis.

Trace amount of contaminate proteins that observed in some TTR fractions obtained from the preparative electrophoresis could be removed out by further purification by affinity chromatography on hRBP-Sepharose. The TTR was applied onto the column that was equilibrated with 50 mM Tris-HCl, pH7.5, 0.5 M NaCl with

a constant flow rate of 0.2 ml/min, followed by washing unbound proteins with ~5 column volume of the buffer. Bound TTR was eluted from the column with distilled water. The eluted fractions were analyzed by native-PAGE followed by silver staining. The TTR containing fractions were pooled, concentrated and kept at -20^{o}C.

2.4 Determination of physicochemical properties of the recombinant TTRs

In order to confirm the proper structure and folding of the TTRs synthesized by *P. pastoris*, the general physicochemical properties of the recombinant TTRs including molecular weight of TTR, mass of the TTR monomer, and the electrophoretic mobility were examined

2.4.1 Molecular weight of TTR tetramer

The molecular weight of TTRs were determined by HPLC/gel-permeation chromatography on BioSil SEC 250 column (300x7.8mm, Bio-Rad), equilibrated with 0.2 M sodium phosphate buffer pH 6.0. The column was standardized with blue dextran (2,000 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kD). Aliquot of the purified TTR (10 to 50 μg) was applied onto the column and separation was conducted at flow rate of 0.4 ml/min. The elution volume (V_e) was followed up from the absorbance at 280 nm. The void volume (V_0) was determined from blue dextran exclusion. The molecular weight was obtained from plots of the partition coefficient K_{av} against log molecular weight. The K_{av} was calculated from the equation:

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where, V_t is the total volume of the column

2.4.2 Subunit mass

Masses of the recombinant TTR subunits were determined by SDS-PAGE, followed the method of Laemmli and Favre (1973). Purified TTR was denatured by mixed with a solution containing 2% sodium dodecyl sulphate (SDS) and 2.5% β-mercaptoethanol and the mixture was boiled for 30 min prior to loading on SDS-polyacrylamide gel **(**12% resolving gel and 4% stacking gel). The separation was performed at a constant voltage (100 volts), then the protein band was detected by staining gel with Coomassie brilliant blue R-250. The relative mobilities of TTR

subunits were calculated and mass of the subunit was determined from a standard curve plotted between R_f and log molecular weights of standard proteins.

2.4.3 Analysis of N-terminal amino acid sequence of TTR

The N-terminal amino acid sequences of the recombinant TTRs were determined by automatic Edman degradation in the commercial facility of the Department of Biochemistry, La Trobe University, Melbourne, Australia, or at Bioservice, NSTDA, Thailand.

2.5 Purification of human TTR from plasma

In order to obtain the purify human TTR for using as a control, the TTR was purified from human plasma by 2 steps of purification, the affinity chromatography on Cibacron blue column (Cibacron Blue 3GA, Sigma) and followed by the preparative native-PAGE.

The first step was carried out by centrifugation of pooled human plasma at 4^{o}C, 10,000 rpm for 20 min to remove any non-dissolved particles. Then the clear supernatant was applied onto a Cibacron blue column that previously equilibrated with 50 mM phosphate buffer, pH 7.4 (PB) at flow rate of 10 ml/h. Thereafter, the column was washed with PB and unbound proteins were collected. The bound proteins were then eluted from column with PB in the presence of 0.5 M NaCl (PBS). Both bound and unbound fractions were analyzed by native-PAGE. Finally, the fractions containing TTR were pooled and concentrated by ultrafiltration.

In the second step of purification, the concentrated pooled TTR fraction was loaded onto a preparative polyacrylamide gel tube (12% resolving gel and 4% stacking gel). The protein separation was performed at constant power (17 Watts), and protein bands were eluted out with 50 mM Tris-HCl, pH 7.4 at a constant flow rate of 1 ml/min. The separation and elution were as described in section 2.3. The presence of TTR in fractions was determined by native-PAGE followed by silver staining. The fractions containing TTR were then pooled and concentrated by ultrafiltration and stored at -20^{o}C.

2.6 Western blot analysis

Proteins were separated by SDS-PAGE or Native-PAGE in duplicate. One of the gel was stained with Coomassie blue while proteins in the other gel were electrophoretically transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Phamacia Biotech) using a vertical tank electrophoresis blotting system (Bio-Rad) at 100 volts, 1 h, for SDS-PAGE or at 25 volts, 30-40 min, for native-PAGE with cooling system. The buffer used was 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3. The protein bands on the membrane were visualized by staining with 0.1% Amido black. Specific proteins were detected by immunochemistry, using enzymatic detection. To block the non-specific binding sites, the membrane was incubated in a blocking solution containing 5% skim milk, 25 mM Tris, pH 7.4, 0.15 M NaCl and 0.1% Tween 20 (TBS-T) at room temperature for 1 h with gentle shaking. Thereafter, the membrane was incubated with a primary antibody (Table 2.2) at an appropriate dilution in the blocking solution at room temperature for 1 h. The excess antibody was removed out by washing with TBS-T at room temperature, 15 min once followed by 5 min for three times. Thereafter, the membrane was incubated with a secondary antibody (Table 2.2) at room temperature for 1 h prior washed again with TBS-T, 15 min once followed by 5 min for three times. The specific protein was detected by enhance chemiluminescence (ECL, GE Healthcare) and the fluorescence signal was detected by Bio-Chemi System (UVP).

2.7 Purification of [^{125}I]-L-thyroxine and [^{125}I]-L- 3,5,3′-triiodothyronine

Commercially available [^{125}I]-thyroxine ([^{125}I]-T_4) and [^{125}I]-3,5,3′-triiodothyronine ([^{125}I]-T_3) always contain free iodine. This free iodine was removed prior to experiments by chromatography using SepPak C18 pre-packed column, as described by Mendel *et al*.(1989). Briefly, 5 µCi of iodinated TH was diluted with 2 ml of methanol, water and phosphoric acid solution (methanol:water 1:1 with 0.3 ml of 85% phosphoric acid per liter) and loaded onto the column that previously washed with 2 ml of methanol and 5 ml of water. Then, the column was washed with methanol, water and phosphoric acid solution (5 ml for [^{125}I]-T_3 purification or 10 ml for [^{125}I]-T_4 purification) to removed free iodine. [^{125}I]- T_4 or [^{125}I]- T_3 was eluted

with 100% methanol. The purity of THs was checked by thin layer chromatography (Pardridge and Mietus, 1980).

2.8 Binding of recombinant TTRs to THs

The TH binding experiment was performed in the Department of Biochemistry and Molecular Biology, University of Melbourn, Australia.

The binding of TH to TTR is quantitatively described by the dissociation constant, K_d. Purified recombinant TTRs (100 nM) was incubated with T_4 or T_3, from 0 to 1000 nM in the presence of tracer amount of [^{125}I]- T_4 or [^{125}I]- T_3 at 4^{o}C, overnight, as described by Chang *et al*. (1999). A volume of 0.4 ml of the incubation mixture was transferred to a vial for total radioactivity determination. Free T_4 or T_3 from the same volume of the incubation mixture was separated from the THs bound by TTR by adsorption to a layer of methyl cellulose coated charcoal on a glass microfilter under constant suction pressure. The charcoal and filter were washed once with 0.4 ml of incubation buffer. The radioactivity was determined using an LKB 1270 Rackgamma II counter with an efficiency of 70%. Non-specific binding can be extrapolated and corrected from the radioactivity of the samples incubated with large amount of unlabeled TH (T_4 or T_3). The dissociation constant (K_d), which quantitatively indicates TH binding to TTR, was calculated from the specific binding and analyzed by Scatchard analysis (Scatchard, 1949).

2.9 Analysis of TTR binding to human RBP

To determine the binding property of TTR to RBP, the purified TTR (0.4 µg) was incubated with various amount of human RBP (0.07 to 5 µg). After incubation at 4°C for 1 h, the reaction mixture was analyzed on a Native-PAGE (10% resolving, 4% stacking) with constant voltage of 100 volt, at 2^{o}C to 4^{o}C. Free TTR and TTR-RBP complex were immunochemically detected by western blotting using antibodies against TTR (Table2.2). The dissociation constant (K_d), which quantitatively indicates binding of TTR to human RBP, was calculated from specific binding and analyzed by Scatchard analysis (Scatchard, 1949). The molecular weights of TTRs used in the K_d determination were directly calculated from the amino acid composition using the

compute pI/MW tool (http://www.expasy.ch/tools/pi_tool.html) as shown in Table 2.3.

2.10 Proteolytic activity of TTRs

To quantify the proteolytic activity of TTRs, two substrates were used; the universal protease substrate, casein, and a specific substrates, apoA-I.

Caseinolytic activity of TTRs was analyzed by the method of Twining (1984) with modifications. Aliquots (50 µl) of a reaction mixture contained 2 µg of purified TTR, 0.2% fluorescein isothiocyanate (FITC)-casein (Sigma), 0.2 M NaCl and 5 mM $CaCl_2$ in 50 mM Tris-HCl, pH8.8 was incubated at 37^{o}C for 12 h in the dark. At each time point, the reaction was stop by adding 120 µl of 5% trichloroacetic acid (TCA). The reactions were allowed to stand at room temperature for 2 h and, then at 4^{o}C for 12 h. The TCA-insoluble protein pellet was sedimented by centrifugation at 12,000 rpm for 10 min. An aliquot (about 150 µl) of the supernatant was diluted with 500 mM Tris HCl, pH 8.5 to make the reaction to a proper pH. The fluorescence was determined using a spectrofluorometer (Shimazu) at an excitation and emission wavelengths of 490 and 525 nm, respectively. At least three replicates of analysis were performed for each TTRs. All reagents and tubes were sterile and a septic technique was used in order to make it confident that the activity was originated from TTR rather than from the contaminated microorganism. The proteolytic activities of the TTRs were calculated using quinine hydrochloride as standard. One relative fluorescence unit equals to the fluorescence of the 6 nM quinine sulfate at an excitation and emission wavelengths of 350 nm and 450 nm, respectively.

For the proteolytic assay using apoA-I as substrate, an equi-molar ratio of TTR and apoA-I (2 µg of TTR and 1 µg of apoAI) were incubated in 50 mM Tris, pH 7.5 at 37^{o}C for various periods. At each time point, the reaction was stop by immediately put the reaction mixture in liquid nitrogen and then kept at -80^{o}C. After complete the time course, the reaction mixture was separated onto 20% SDS-PAGE at 4^{o}C with a constant voltage (180 volts). The protein bands were visualized by staining with Coomassie blue and then the cleaved and uncleaved apoAI were determined from intensity of the protein bands detected by a Gel document using Lab Works version 4.6 (Bio-Imagine System ,UVP).

2.11 Preparation of competent cell and transformation of DNA

2.11.1 Preparation of *E. coli* competent cell

The competent cells were prepared from *E. coli* strain DH5α using calcium chloride as described by Cohen *et al.* (1992). A single colony from fresh LB agar plate was inoculated into 5 ml of LB broth in a 50-ml tube and grown overnight at 37°C in an orbital shaking incubator (150 rpm). Then, 50 µl of the overnight culture was transferred to 25 ml of LB broth and cells were grown until the OD_{600} reached 0.4 to 0.5 (took 2 to 3 h). Then, cells were cooled down to 0^oC by storing on ice for 10 min and collected by centrifugation at 2,500 rpm, 4^oC, for 10 min. The supernatant was removed and tube was inverted for 1 to 2 min to drain any trace of medium. Cell pellets were suspended in 4 ml of ice-cold 0.1 M calcium chloride and cooled down to 0°C. After centrifugation and draining, cells were suspended in 0.8 ml of ice-cold 0.1 M calcium chloride. Thereafter, the competent cells were aliquot (100 µl) and 60% glycerol was added to the final concentration of 15.5% (v/v). Immediately, cells were frozen in liquid nitrogen and stored at -80°C as a glycerol stock.

To transform a plasmid, 50 µl of the *E. coli* competent cell suspension was gently mixed with 5 µl of the DNA plasmid in a polypropylene tube and stored on ice for 10 min. Then, the cell mixture was subjected to heat-shock for exactly 90 s at 42^oC without shaking and cooled down to 0^oC for 1 to 2 min. Thereafter, 100 µl of SOC medium was added and the mixture was incubated at 37^oC for 1 h prior to plating onto LB agar plate, in the presence 25 µg /ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and ampicillin (100 µg/ml agar). Finally, plates were incubated at 37°C for 16-18 h.

2.11.2 *Pichia* competent cell

To prepare the yeast competent cell, a single colony of *P. pastoris* strain GS115 or SMD 1168 grown on the yeast extract peptone dextrose (YPD) agar plate was transferred to 5 ml of YPD broth and cells were grown at 30^oC, overnight. Then, 100 µl of the overnight culture was transferred into 120 ml of fresh YPD medium in a 1-liter flask and continuously grown to an OD_{600} of 1 to 1.6. Cells were collected by centrifugation at 2,000 rpm, 4^oC, for 5 min prior the cell pellet was

suspended in 45 ml of the ice-cold sterile distilled water. Cells were collected by centrifugation and resuspended once in 22.5 ml of the ice-cold sterile distilled water. Thereafter, cells were resuspended in 1.8 ml of ice-cold 1.0 M sorbitol, and finally resuspended in 0.135 ml of the ice-cold sorbitol. The competent cells were aliquot, kept at -80^{o}C or immediately used in transformation.

2.12 Purification of PCR product

In order to obtain a purified PCR product for further study, the DNA fragment amplified by PCR was purified by using a QIAquick PCR Purification Kit (QIAGEN) or by separation on an agarose gel followed by extraction the DNA with QIAquick Gel Extraction kit (QIAGEN).

To purify the PCR product by using the QIAquick PCR Purification Kit, 5 volumes of the binding buffer were mixed with a solution containing the PCR product, then the entire mixture was applied onto a QIAquick spin column. The impurities were washed out with a buffer containing ethanol. In final step, the DNA that bound to the column was eluted out with 30 to 50 µl of TE and stored at -20°C.

The DNA that was separated on an agarose gel was extracted and purified from gel by using the QIAquick Gel Extraction kit (QIAGEN). After DNA separation on 1% agarose containing 0.4 µg of ethidium bromide, the DNA band was excised with a sterile razor blade. Three volumes of the solution containing quanidine thiocyanate (QG buffer) were added to 1 volume of the gel slice and the reaction was incubated at 50^{o}C for 10 min, with vortex mixing every 3 min. Thereafter, 1 gel volume of isopropanol was added, and the entire mixture was applied onto a QIA spin column. The DNA adsorbed onto membrane in the column while other impurities were washed out with 750 µl of a buffer containing ethanol. The purified DNA was eluted out from the column with 30 µl of 10 mM Tris-HCl, pH 8.5 and stored at -20^{o}C until used.

2.13 Purification of plasmid

2.13.1 by using the QIAprep Spin kit

The *E. coli* plasmid was prepared by the alkaline lysis described by Birnboim and Doly (1979), using a QIAprep spin kit. An overnight culture of the plasmid was prepared in the presence of antibiotic (50 μg ampicillin/ml of culture).

The bacterial cells were collected by centrifugation at 10,000 rpm for 15 s, and resuspended in 250 μl of 50 mM TE and RNase A (buffer P1). Then, the bacterial membrane was disrupted with 250 μl of a lysis buffer containing NaOH for 5 min at room temperature. The cell lysate was neutralized and adjusted with guanidine hydrochloride, a chaotropic salt, to a concentration of 1.6 M. Any precipitates of the bacterial chromosomal DNA and cell debris were removed by centrifugation at 14,000 rpm for 10 min. Then, plasmid in the supernatant was purified by absorption onto the surface of silica filter in a spin column. Bound plasmid was then separated from salts, proteins and other cellular impurities by washing with 750 μl of 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 in 80% methanol. The plasmid was finally eluted with 50 μl of 10mM Tris-HCl, pH 8.5, and stored at -20^{o}C until used.

2.13.2 by using the PCR purification kit

A plasmid in an enzymatic reaction was purified with the same method described for purification of PCR product in section 2.12, using a QIAquick PCR Purification Kit (QIAGEN).

2.14 Purification of DNA by phenol-chroloform extraction

The purified plasmid or the ligated DNA that were used for transformation was further purified by phenol-chloroform extraction in order to concentrate and/or obtain higher purity. An equal volume of a plasmid DNA in TE and a solution containing phenol/chloroform/isoamyl alcohol (25:24:1) was vigorously mixed by vortexing prior to centrifugation at 14,000 rpm for 15 min. The clear aqueous phase was transferred to a new tube and 0.1 volume of 3 M sodium acetate, pH5.2 and 2 volume of ice-cold ethanol were added. The solution was mixed and left at -80^{o}C for 2 h or overnight to precipitate the DNA completely. Thereafter, the solution was centrifuged at 14,000 rpm for 15 min and the DNA pellet was washed once with 70%

ethanol prior to dry up at room temperature. Finally, the DNA pellet was dissolved in a small volume of TE and stored at -20^{o}C.

2.15 Digestion of plasmid or DNA fragment with restriction endonucleases

In order to determine the presence and size of the inserted DNA in a plasmid or generate the restriction sites of a DNA fragment, single or double digestion with appropriate restriction endonucleases was performed. The digestion was carried out in 20 µl of the reaction mixture containing 1 to 4 µg of DNA, 1 to 10 units of each restriction enzyme and appropriate reaction buffer. The reaction was allowed to occurred at 37^{o}C for 1 to 2 h. To determine an inserted DNA, the reaction mixture was analyzed by electrophoresis on 1% agarose gel containing 0.4 µg/ml of ethidium bromide.

2.16 Preparation of anti-crocTTR polyclonal antibody

To prepare a polyclonal antibody raised against crocTTR for use to determine the binding ability of TTRs to human RBP, a male rabbit (Albino) (2 years old) was immunized twice with recombinant crocTTR (150 µg per immunization; dissolved in 50 mM Tris-HCl, pH7.5, 0.15 M NaCl), mixed with 50% Freund's adjuvant (Sigma). After 3 weeks, the first booster was given with 150 µg of the recombinant crocTTR mixed with 50% incomplete Freund's adjuvant (Sigma). After the first immunization for 2 weeks, a small blood sample was collected and the serum was detected a specific titer by Ouchterlony double immunodiffusion (Ouchterlony, 1956). Once a high specific titer was obtained (after boosted 3 times over 6 weeks), blood was collected for larger volume (15-20 ml). The serum was prepared and the antibody was partially purified according to the method of Warden and Giese (1984). In brief, the serum was precipitated with 50% ammonium sulfate, centrifuged at 12,000 rpm for 40 min. The pellet was dissolved in 50 mM Tris-HCl, pH7.5 and dialyzed against the buffer to remove salt. The dialysate was centrifuged to separated out any un-dissolved particles prior the clear supernatant containing antibody was aliquot and stored at -20^{o}C until used.

2.17 SDS-PAGE and non-denaturing PAGE (native-PAGE)

The separation of proteins under denaturing condition was carried out by sodium dodecyl sulfate polyacrylamide gel electrphoresis (SDS-PAGE) using 12% or 20% polyacrylamide resolving gel (pH 8) and 4% polyacrylamide stacking gel (pH 6.8) with the discontinuous buffer system of Laemmli and Favre (1973). Protein samples mixed with a solution containing SDS (69 mM) and β-mercaptoethanol (320 mM), and incubated at 100°C for 30 min or 5 min (for standard proteins) prior the proteins were loaded onto gel. The electrophoretic separation was carried out in the buffer containing 25mM Tris, 192 mM Glycine, 0.1%SDS, pH 8.3. The protein bands were detected by staining gel with silver nitrate (Morrissey, 1981) or Coomassie Brilliant blue R-250.

The separation of proteins under non-denaturing condition was carried out as the same that described for SDS-PAGE, except no SDS was added and 10% resolving and 4% stacking gels were used. To detect protein bands, gel was stained with Coomassie Brilliant blue R-250 or silver nitrate.

2.18 Protein staining

2.18.1 by Coomassie blue staining

After electrophoresis, gel was immersed in 0.2% Coomassie brilliant blue R-250, dissolved in 50% methanol and 10% acetic acid, for 1 h. Excess dye was removed by rinsing the gel in a solution of 50% methanol and 7.5% acetic acid until background was clear. For storage, gel was kept in a solution of 5% methanol and 7.5% acetic acid.

2.18.2 by silver staining

Gel staining with silver nitrate was performed by the method described by Morrissey (1981). After electrophoresis, gel was immersed in the solution of 45% methanol and 10% acetic acid for 30 min and then transferred to the solution of 7.5% acetic acid and 5% methanol for 30 min. Thereafter, gel was rinsed with distilled water for 3 to 4 changes prior soaking in a solution of dithiothreitol (DTT) (500 µg/ml) for 30 min. The DTT solution was discarded and gel was soaked in 0.1% silver nitrate for 20 min. To develop, gel was immersed in a solution of 3% (v/v) sodium carbonate and 0.02% (v/v) formaldehyde until protein bands

appeared (took ~2-5 min depending on amount of proteins). Then, small amount of 50% acetic acid was added (until a few air bubbling occurred) to stop the reaction. Gel was washed several times with distilled water, and stored at 4^{o}C. The gel stained was photographed and band intensity was analyzed by gel document with LabWork 4.0 or 4.6 version (UVP).

2.19 Determination of protein concentration

Protein content was determined using Bradford protein assay (Bradford, 1976). The assay solution (1 ml), which comprised of 0.085 mg/ml Coomassie blue G-250, 5% methanol and 5.06% H_3PO_4, was added into 0.1 ml of a protein solution. The protein-dye complex formation was allowed at room temperature for 2 min prior the optical density at 595 nm of the reaction mixture was measured. Bovine serum albumin (BSA) was used as standard.

Table 2.1 **Oligonucleotide primers used to generate cDNA for recombinant TTRs**

TTR cDNA	Vector	PCR step	Sequence 5′ → 3′	Sense
huTTR	pPIC3.5	1	AGGATCCAGGATGGCTTCTCATCG	Sense
			AGGAGTGAATTCTCATTCCTTGGGATTGG	Antisense
	pPIC9	1	TCTCGAGAAAAGAGAGGCTGAAGCTGGCCCTACGGGG	Sense
			AGGAGTGAATTCTCATTCCTTGGGATTGG	Antisense
hu/crocTTR	pPIC9	1	AACGGGCACCGGTGAATCCAAATGCC	Sense
			ACGGAATTCTTATTCTTGTGGATCACTG	Antisense
		2	CTCGAGAAAAGAGAGGCTGAAGCTGGCCCAACGGGCACCGG	Sense
			ACGGAATTCTTATTCTTGTGGATCACTG	Antisense
croc/huTTR	pPIC9	1	ACTGGTTTCCCATGGTTCTATTGATTCCAAGTGTCC	Sense
			AGGAGTGAATTCTCATTCCTTGGGATTGG	Antisense
		2	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
			AGGAGTGAATTCTCATTCCTTGGGATTGG	Antisense
truncated crocTTR	pPIC9	1	CTCGAGAAAAGATCCAAATGCCCACTTATGG	Sense
			ACGGAATTCTTATTCTTGTGGATGACTG	Antisense
pigC/crocTTR	pPIC9	1	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
			TGGGGCTGCTGACGAGGGCTGTG	Antisense
		2	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
			AGCTCCCTCCTTGGGGCTGCTGAC	Antisense
		3	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
			ACGGAATTCTCAAAGAGCTCCCTCCTTGGG	Antisense

Table 2.2 Antibodies used for Western blot analysis

TTR	Primary antibody and dilution used	Secondary antibody and dilution used
crocTTR and its chimeras: hu/crocTTR truncated crocTTR xeno/crocTTR pigC/crocTTR	anti-crocTTR polyclonal antibody, raised in rabbit (dilution 1:500)	Rabbit IgG-HRP, raised in goat (dilution 1:2500)
plasma huTTR, recombinant huTTR and croc/huTTR	anti-human TTR polyclonal antibody, raised in sheep (dilution 1:2500)	Sheep IgG-HRP, raised in rabbit (dilution 1:2500)

Table 2.3 Molecular weights of TTRs

(Direct calculated from the amino acid sequences of TTRs by the compute pI/MW tool , http://www.expasy.ch/tools/pi_tool.html).

The TTRs those were underlined were synthesized in this thesis. While the others were the available clones in laboratory.

TTR	Amino acid sequence	MW (kDa)
Plasma huTTR	residue Gly1 to Glu127	55.17
croc/huTTR	residue Ala1 to Asp7 of crocodile TTR and residue Ser8 to Glu 127 of human TTR	56.56
crocTTR	residue Ala1 to Glu127	56.88
hu/crocTTR	residue Gly1 to Glu7 of human TTR and residues Ser8 to Glu127 of crocodile TTR	55.37
xeno/crocTTR	residue Ala1 to Ser8 of xenopus TTR and residue Lys9 to Glu 127 of croc TTR	57.48
truncated crocTTR	residue Ser8 to Glu127 of crocodile TTR	52.97
pigC/crocTTR	residue Ala1 to Ala120 of crocodile TTR and residue Leu121 to Leu130 of pigTTR	57.78

CHAPTER 3

RESULTS

PART I: EXPRESSION OF THE RECOMBINANT TTRS IN A METHYLOTROPHIC YEAST, *PICHIA PASTORIS*

1. Construction of *Pichia* clone of recombinant wild type TTR and recombinant chimeric TTRs

To date, the primary structure of TTRs, either partial or full length, has been analyzed. The alignment of the vertebrate TTR amino acid sequences revealed that residues in all 17 positions in the central channel including those involved in the binding interaction with THs were conserved (Blake and Oatley, 1977; Blake, 1981 and Wojtczak *et al*., 1996). In the contrary, the predominant changes concentrated at the N-terminal region and lesser changes on the C-terminal region of the TTR subunit. The change of length and hydropathy of these regions during evolution are correlated to the change in binding affinities to T_4 and T_3 (as reviewed in Chapter 1 section 3). Since these two segments locate at the entrance to the channel where the binding sites for THs exist, the question arises whether both or either of these segments influences on the accession of THs or other ligands to the intramolecular binding sites of TTR. To determine influence of N- and C-termini, the synthesis of the recombinant chimeric TTR in which the N- or C-terminal region was replaced by that of other TTRs with different binding favor to THs is the choice.

In this thesis, several types of the recombinant wild type TTRs and recombinant chimeric TTRs were used for studying the influence of N- or C-terminal regions on functions of TTR. These recombinant TTRs include two wild type TTRs: the human TTR (recombinant huTTR) and *C. porosus* TTR (crocTTR), one chimeric human TTR: the human TTR with the N-terminal segment of *C. porosus* TTR (croc/huTTR), and four chimeric *C. porosus* TTR: the *C. porosus* TTR with the N-terminal segment of human TTR (hu/crocTTR), the *C. porosus* TTR with the N-terminal segment of *X. laevis* TTR (xeno/crocTTR), the *C. porosus* TTR with the C-terminal segment of pig

TTR (pigC/crocTTR) and the *crocodile* TTR without N-terminal segment (truncated crocTTR). Some recombinant clones are available in the laboratory and some were newly constructed in this thesis.

The newly clones were recombinant huTTR, croc/huTTR, hu/crocTTR, pigC/crocTTR and truncated crocTTR. The recombinant huTTR was synthesized in order to confirm similar properties of the recombinant TTRs produced by *P. pastoris* as the native TTR in particulr the binding to THs. The recombinant wild type crocTTR was previously synthesized in our laboratory due to the fact that the *C. porosus* synthesized TTR in liver only during development, and synthesized the TTR in choroids plexus when the animal becomes adult (Prapunpoj *et al*., 2002). Therefore, synthesis of the recombinant crocTTR can provide sufficient amount of the TTR for further studies.

To construct the *Pichia* recombinant clone for huTTR, two expression vectors were used. These were pPIC3.5 in which the recombinant protein will be extracellularly secreted under itself presegment sequence and pPIC9 in which the recombinant protein will be extracellularly secreted under presegment sequence of the *Pichia* α-factor protein (Kurjan and Herskowitz, 1982). Human TTR cDNA was amplified and generated the compatible restriction ends for ligation into the expression vectors (*Bam*HI at 5′ end and *Eco*RI at 3′ end for ligation into pPIC 3.5 and *Xho*I at 5′ end and *Eco*RI at 3′ end for ligation into pPIC9) by PCR using the primer sets as shown in Table 2.1. By PCR, the DNA product with an expected size of ~400 bp could be obtained (Figure 3.1). The PCR product was purified and double digested with the appropriate restriction enzymes prior to being ligated into the expression vector that was predigested with the same enzymes. In pPIC3.5, human TTR cDNA was placed under the control of the native AOX1 at *Bam*HI and *ECo*RI sites without any modification and the native presegment sequence was used for secretion of the TTR (Figure 3.2A). Whereas, in pPIC9, the human TTR cDNA with the compatible restriction sites for *Xho*I and *Eco*RI was placed behind the yeast α-factor signal sequence (Figure 3.2B). The α-factor signal sequence was used in the gene expression and removed within golgi apparatus by KEX2, the diabasic amino acid sequence recognizing protease (Julius *et al*., 1984a and 1984b). The sequence

Glu-Lys-Arg is necessary for the release process of α-factor peptide from the gene product by KEX2, and the cleavage by KEX2 occurs between Arg and Gln in the sequence Glu-Lys-Arg-Glu-Ala-Glu-Ala (Brake *et al*., 1984). The Glu-Ala-Glu-Ala sequence is necessary for the correct cleavage and will be removed during translocation of the recombinant protein. Therefore, the human TTR cDNA was constructed into pPIC9 so that the cDNA was immediately in-frame integrated with the coding portion of Glu-Ala-Glu-Ala.

The cDNA coding for croc/huTTR was constructed and amplified by PCR using human TTR cDNA as template. Whereas, the cDNAs coding for hu/crocTTR, pigC/crocTTR and truncated crocTTR were generated using *C. porosus* TTR cDNA as template, as described in the Materials and Methods section 2.1.1. Pairs of the specific primers (Table 2.1) were used to alter the N- or C-terminal sequence and generate the recognition sequences for *XhoI* at 5' and *ECo*RI at 3' ends in the TTR template. The PCR product with an expected size (~400 bp) could be obtained from each reaction. These DNAs were purified and double digested with *Xho*I and *EcoR*I prior to being ligated at the *Xho*I and *Eco*RI sites of pPIC9. In similarly to huTTR, the DNA fragments of all these chimeric TTRs were placed adjacent to the α-factor signal sequence of pPIC9 as show in Figure 3.3.

Prior transformations into *P. pastoris*, the recombinant expression plasmids were analyzed by nucleotide sequencing to confirm the sequences and the insertion direction of the TTR cDNAs in the expression vector. The sequencing results confirmed the cDNAs were correctly placed into the vectors. In addition, the nucleotide sequences of the cDNAs were confirmed and, as expected, would code for the desired amino acid residues as shown in Table 2.3. Thereafter, the *Pichia* expression vectors were linearlized by *Sal*I prior to being introduced into *P. pastoris* strain GS115 or SMD 1168 by electroporation. Since histidinal dehydrogenase gene (*HIS*4) of these two *Pichia* strains were mutated (Cregg *et al*., 1985), so that the *Pichia* strain can use methanol but requires hitidine supplementation for growth (genotype his4, phenotype $Mut^{+}His^{-}$). In order to create the His^{+}/Mut^{+} recombinant that has ability to synthesize histidine and utilize methanol, crossing over between the *HIS*4 gene on the plasmid and the *his*4 locus of the yeast chromosome is required (Cregg *et al*., 1985 and Cregg *et al*., 1989). As the consequence, the *Pichia* cell that

obtains an expression vector will have a His^+ phenotype. By electroporation, approximate $2x10^3$ to $5x10^3$ colonies of the *Pichia* transformants with His^+ phenotype were obtained per microgram of plasmid. This transformation efficiency was similar to that previously reported by Scorer *et al*., 1994 in which 10^3 to 10^4 colonies of His^+ were obtained per microgram of DNA.

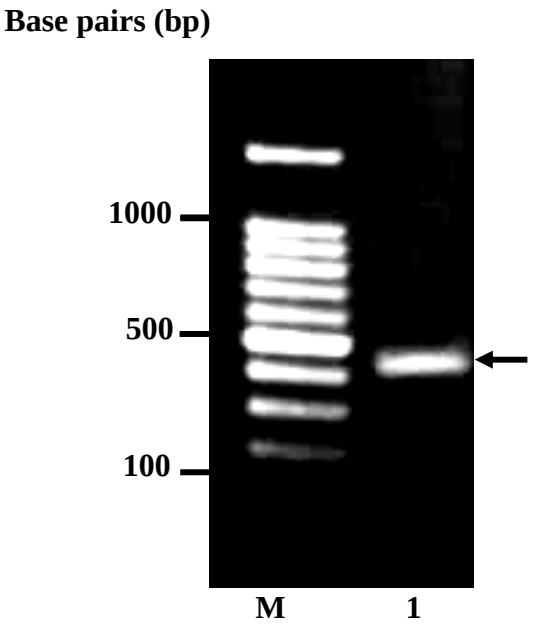
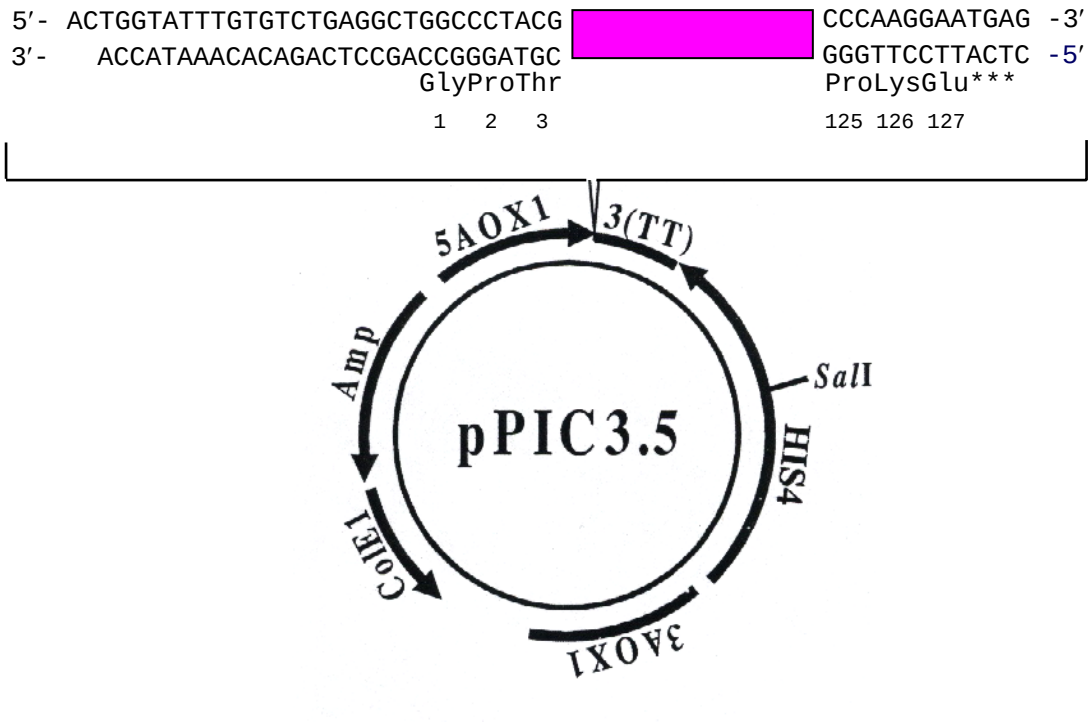


Figure 3.1 Generation and amplification of TTR cDNA by PCR

The cDNA coding for pigC/crocTTR was generated and amplified by PCR using *C. porosus* TTR cDNA as template. The PCR product was analyzed on agarose gel (1%) in the presence of ethidium bromide. DNA bands were visualized under UV. A single band of the DNA with approximate 400 bp was indicated by arrow. M is 100 bp DNA markers. Sizes of the DNA markers were also shown.

A


5′- ACTGGTATTTGTGTCTGAGGCTGGCCCTACG
3′- ACCATAAACACAGACTCCGACCGGGATGC
GlyProThr
1 2 3
CCCAAGGAATGAG -3′
GGGTTCCTTACTC -5′
ProLysGlu***
125 126 127
5AOX1
3(TT)
Amp
SalI
HIS4
pPIC3.5
ColE1
3AOX1

B

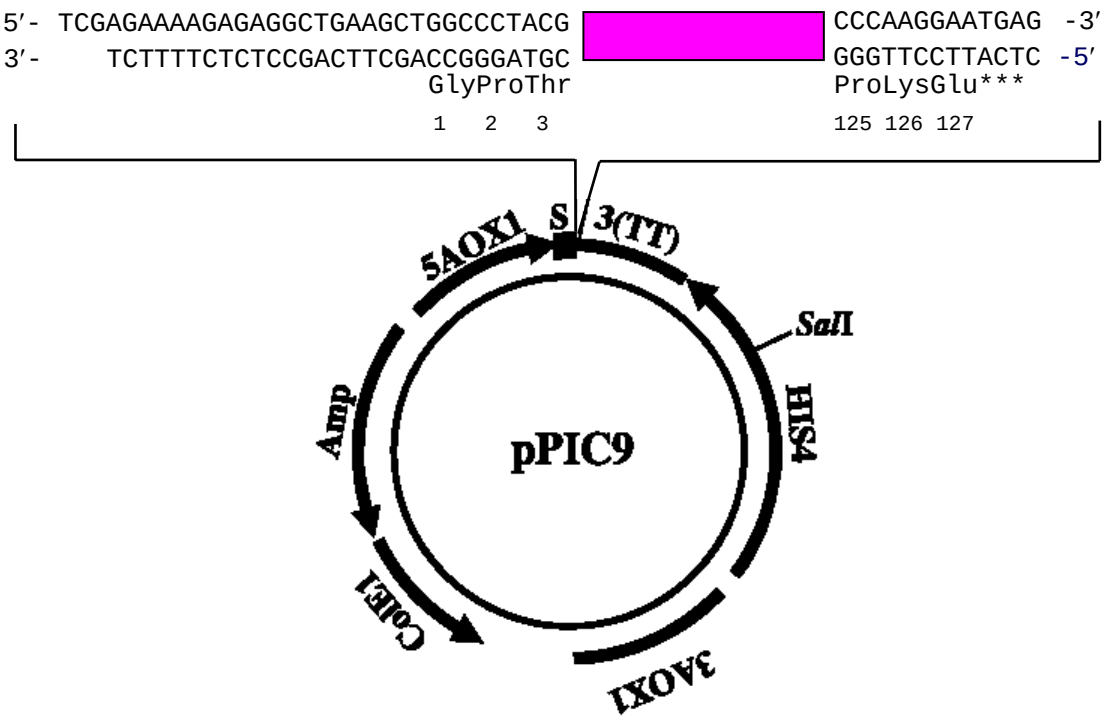

5′- TCGAGAAAAGAGAGGCTGAAGCTGGCCCTACG
3′- TCTTTTCTCTCCGACTTCGACCGGGATGC
GlyProThr
1 2 3
CCCAAGGAATGAG -3′
GGGTTCCTTACTC -5′
ProLysGlu***
125 126 127
5AOX1
S
3(TT)
SalI
Amp
HIS4
pPIC9
ColE1
3AOX1

Figure 3.2 Construction of the expression vectors for wild type human TTR

The expression vectors were constructed from pPIC3.5 (A) and pPIC9 (B) using the human the TTR itself presegment sequence and the α-factor signal sequence for secretion of the recombinant TTR, respectively. The diagrams showed the nucleotide and the derived amino acid sequences at the 5′ and 3′ ends of human TTR after the recognition sites for *Bam*HI and *Eco*RI or for *Xho*I and *Eco*RI were introduced. 5AOX, promoter of alcoholoxidase1 gene in *P. pastoris*; 3(TT), native transcription termination and polyadenylation signal of the alcohol oxidase1 gene; 3AOX1, sequence from the alcohol oxidase1 gene, 3′ to the TT sequences; HIS4, histydinol dehydrogenase gene; Amp, ampicilin resistance gene; ColE1, *E. coli* origin of replication; S, α-factor secretion signal (269 bp); *Sal*I, restriction site for linearization of the vector by *Sal*I.

A

```
5′- AAAAGAGAGGCTGAAGCTGCCCCACTG          CCCAAGGAATGAG -3′
3′- TTTTCTCTCCGACTTCGACGGGGTGAC          GGGTTCCTTACTC-5′
                    AlaProLeu                    ProLysGlu****
                      1  2  3                   125 126 127
```

B

```
5′- TCGAGAAAAGAGAGGCTGAAGCTGGCCCTACG          CCACAAGAATAA -3′
3′-    TCTTTTCTCTCCGACTTCGACCGGGATGC          GGTGTTCTTATTC-5′
                    GlyProThr                    ProGlnGlu***
                      1  2  3                   125 126 127
```

C

```
5′- TCGAGAAAAGATCCAAATGCCCACTTATG          CCACAAGAATAA -3′
3′-    TCTTTTCTAGGTTTACGGGTGAATAC          GGTGTTCTTATTC-5′
                 SerLysCysProLeuMet              ProGlnGlu***
                   8  9  10 11 12 13             125 126 127
```

D

```
5′- TCTCTCGAGAAAAGAGCCCCACTG          GGAGCTCTTTGA -3′
3′- AGAGAGCTCTTTTCTCGGGGTGAC          CCTCGAGAAACT -5′
                    AlaProLeu                    GlyAlaLeu***
                      1  2  3                   128 129 130
```

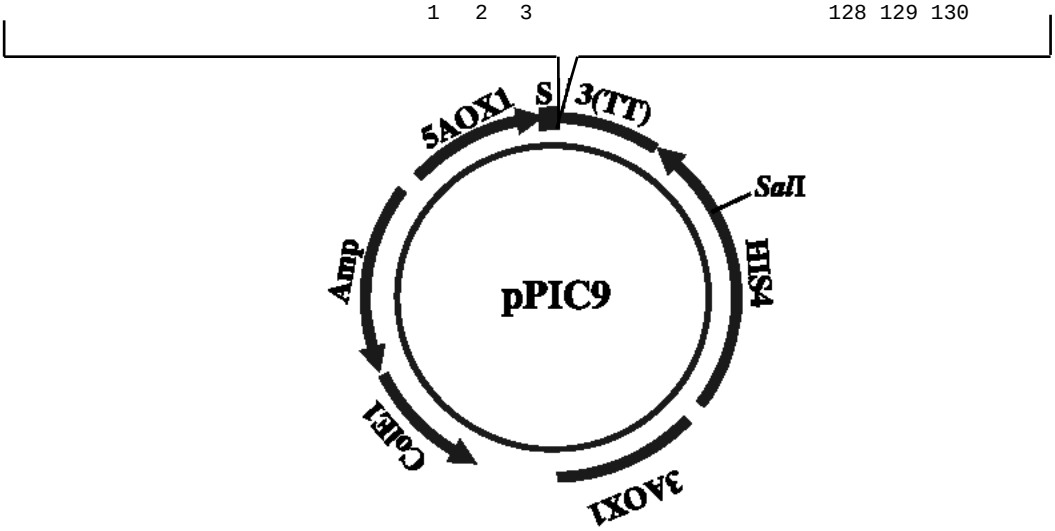


Figure 3.3 The recombinant expression vectors for chimeric TTRs

The recombinant expression plasmids for (A) croc/huTTR, (B) hu/crocTTR (C) truncated crocTTR and (D) pigC/crocTTR were constructed in pPIC9. The recombinant proteins were synthesized under the control of AOX1 promoter and be secreted using the presegment sequence of the α-factor protein of *Pichia*. Pink box, fragment of human TTR cDNA; grey box, fragment of *C. porosus* TTR cDNA; yellow box, fragment of pig TTR cDNA; 5AOX, promoter of alcoholoxidase1 gene in *P. pastoris*; 3(TT), native transcription termination and polyadenylation signal of alcohol oxidase1 gene; 3AOX1, sequence from the alcohol oxidase1 gene, 3 to the TT sequences; HIS4, histydinol dehydrogenase gene; Amp, ampicilin resistance gene; ColE1, *E. coli* origin of replication; S, α-factor secretion signal (269 bases); *Sal*I, Sal I restriction site for linearization of the vector. Numbering of the amino acid residues was based on that for human TTR (Aldred *et al*., 1997).

2. Screening for His^+Mut^+

The heterologous protein expression in *P. pastoris*, used in this thesis, had the *AOX*1 promoter to drive an expression of the TTR genes. However, there is a possibility that cell would lose the *AOX*1 gene while transformation, and can lead to the strain methanol utilization slow or Mut^s that exhibit poor growth on methanol media due to the absence of alcohol oxidase activity. The wild type (Mut^+ or methanol utilization plus) transformants that have ability to metabolize methanol could be differentiated from the Mut^s transformants by comparing growth of the *Pichia* transformants on the minimal methanol medium (MM) and the minimum dextrose medium (MD). Screening for the His^+Mut^+ clones was performed with 80 to 100 His^+ transformants. After incubation for 3 days, growth of the transformants was observed. It was found that most of the *Pichia* His^+ transformants obtained had His^+Mut^+ phenotype (Figure 3.4). Growth of the *Pichia* clones were similar on both MM and MD plate. Up to 50 single colonies of each TTR His^+Mut^+ transformants were selected for the induction synthesis of the recombinant TTRs.

3. Synthesis of recombinant TTRs

3.1 Small scale synthesis

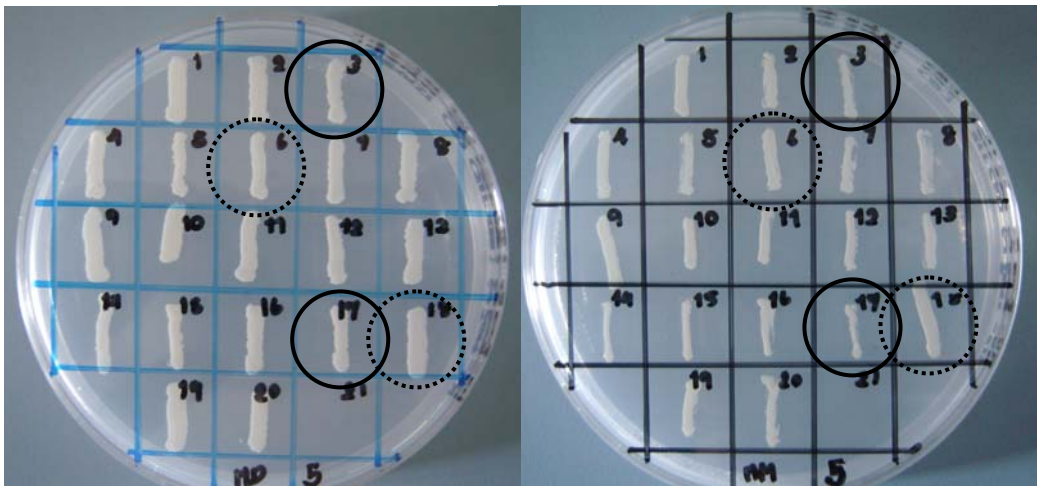
The synthesis for the recombinant TTRs were first attempted in a small scale so that the transformant with the highest production of TTR could be selected and optimization of the synthesis condition could be pursued. The *Pichia* His^+Mut^+ clones were induced with 0.5% methanol for synthesis in 5 ml of culture medium at 30°C for 3 to 5 days. Aliquot of the culture supernatant were analyzed by SDS-PAGE on 12% resolving and 4% stacking gels. By silver staining, it revealed that the recombinant huTTR could be synthesized from the cDNA constructed in pPIC3.5 but not in pPIC9 (Figure3.5). The protein had a subunit mass of about 17 kDa by SDS-PAGE, which corresponded to that of the TTR purified from human plasma. Only one of the recombinant huTTR clones that provided the highest production level of the TTR was selected for larger scale production. For the recombinant chimeric TTRs, high synthesis levels of the recombinant proteins corresponding to TTRs were also obtained. The subunit mass by SDS-PAGE was approximate 17 kDa for croc/huTTR

and 14 kDa for hu/crocTTR, pigC/crocTTR and truncated crocTTR. The production of pigC/crocTTR was shown in Figure3.6.

Since the *Pichia* transformants of each chimeric TTR produced in similar amount of the protein, only one recombinant clone was selected for synthesis in larger amount.

3.2 Large scale production of the recombinant TTRs

In order to obtained sufficient amount of the recombinant TTRs for further studies, all types of the TTR clones both new constructed clones and the available clones were subjected to the protein synthesis in large scale. The shaking flask method was selected for the production since it needs simple equipments. In this method, the synthesis was carried out in 300 ml of BMMY using 2000 ml-flask. Aliquots of the culture were collected every 24 h and resolved by native-PAGE (10% resolving and 4% stacking gels). The secreted proteins were detected by silver staining. Kinetic of the TTR production and secretion from each type of the recombinant clone was shown in Figure 3.7. The results revealed the presence of the expected protein that migrated with the mobility similar to the TTR in human plasma. All recombinant TTRs first appeared after induction with 0.5% methanol for 24 h and reach maximum after 72 h of the induction. No degradation of the TTRs, in particular huTTR, was detected after induction for 72 h. This indicated the stability of the recombinant TTRs produced by *P. pastoris*. This was similar to that previously reported for other recombinant TTRs produced by using the heterologous protein expression system of *P. pastoris*. These include the native TTR from Shrew (Prapunpoj *et al*., 2000a), *X. laevis* (Prapunpoj *et al*., 2000b) and *C. porosus* (Prapunpoj *et al*., 2002). The production yield of all recombinant TTRs varied as shown in Table 3.1. Most of the recombinant clones except truncated crocTTR showed higher production level than the clone for recombinant huTTR. Sixty to two hundreds milligrams of the recombinant TTRs could be synthesized and secreted in 1 liter of culture medium, while the production of the recombinant huTTR was 9 mg per 1 liter of the culture. The recombinant clone for the truncated crocTTR provided the smallest amount of the recombinant protein. Only ~3 mg of the truncated crocTTR could be obtained from 1 liter of culture.



MD **MM**

Figure 3.4 Screening for His^+Mut^+ transformants

The phenotype His^+Mut^+ can be differentiated from His^+Mut^s by comparing growth of the *Pichia* His^+ transformant between on MM and MD plates. The His^+Mut^+ clones, but not the His^+Mut^s, can grow well on both media. Circle and dot circle represent the His^+Mut^s and His^+Mut^+ transformants, respectively.

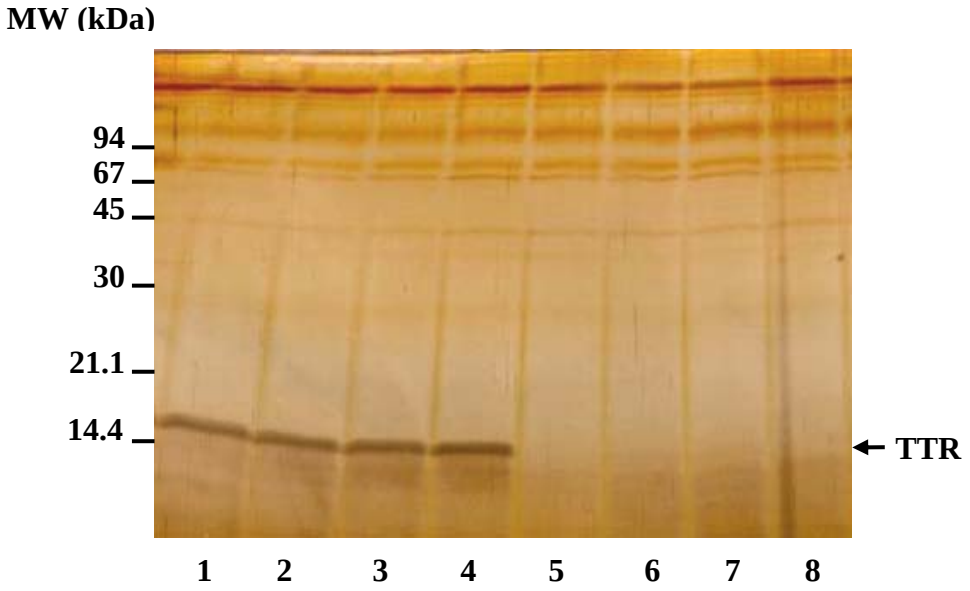


Figure 3.5 Expression of recombinant huTTR

Pichia transformant clones containing human TTR cDNA inserted in pPIC3.5 and pPIC9 were grown and induced with methanol for 4 days. Supernatant of the yeast culture was collected and aliquot of 90 µl were analyzed by SDS-PAGE (15% resolving gel) and protein bands were detected by silver staining. Positions of protein markers and TTR are indicated. Numbers under lanes represent individual clones with DNA inserted in pPIC3.5 (lane1-4) and pPIC9 (lane 5-8).

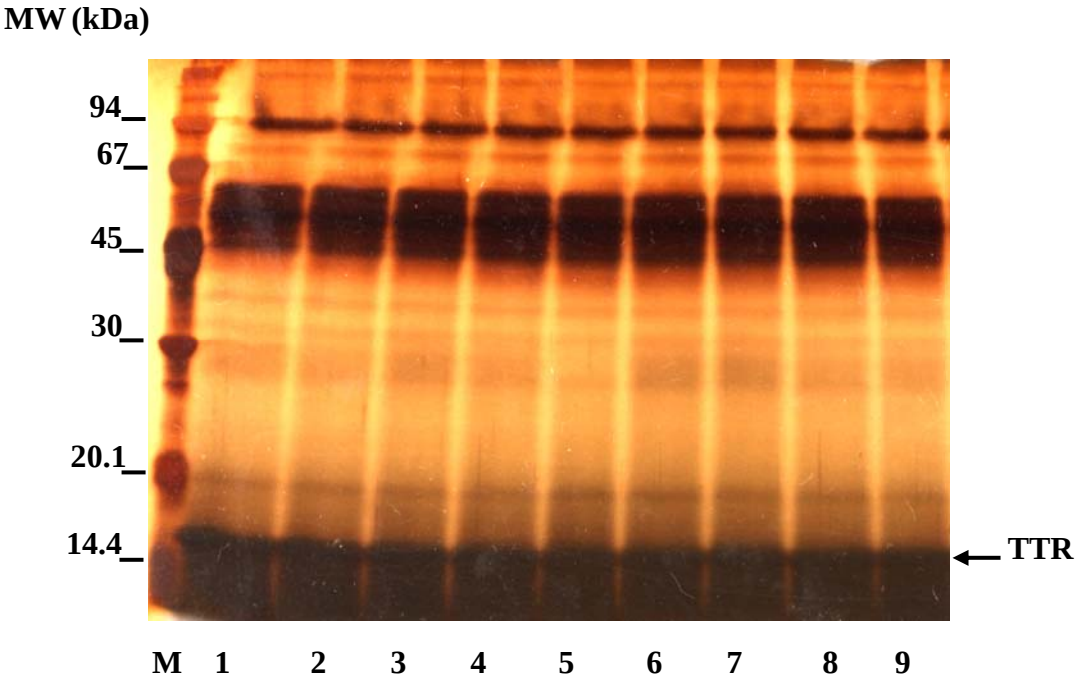
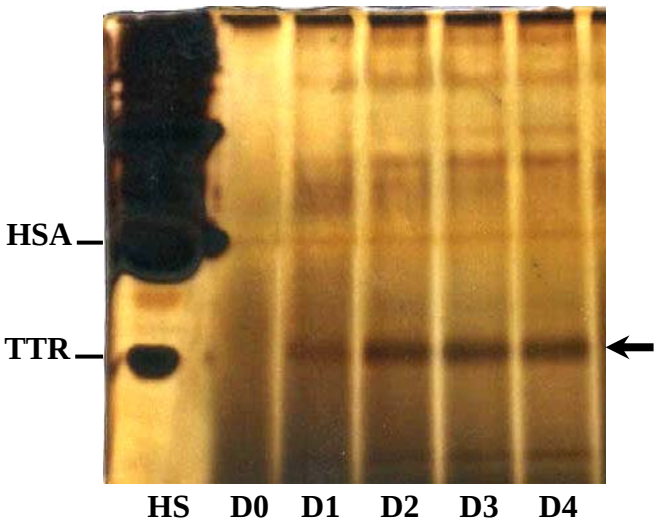


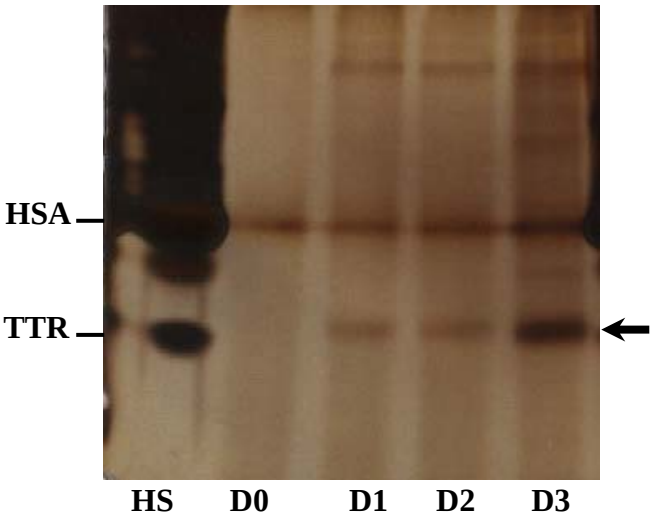
Figure 3.6 Small scale expression of the pigC/crocTTR

Pichia transformants with phenotype $His^{+}Mut^{+}$ up to 50 colonies were individually grown in BMMY and induced for protein synthesis with 0.5% methanol for 3 days. Aliquot of the culture medium of the third day of induction was collected and analyzed for synthesis level of the recombinant protein. Aliquot (50 µl) of the culture supernatant was resolved by SDS-PAGE (12% resolving and 4% stacking) followed by silver staining. M, low molecular weight protein matkers; 1 to 9, an individual *Pichia* clone. Position of the expected band of pigC/crocTTR was indicated by arrow.

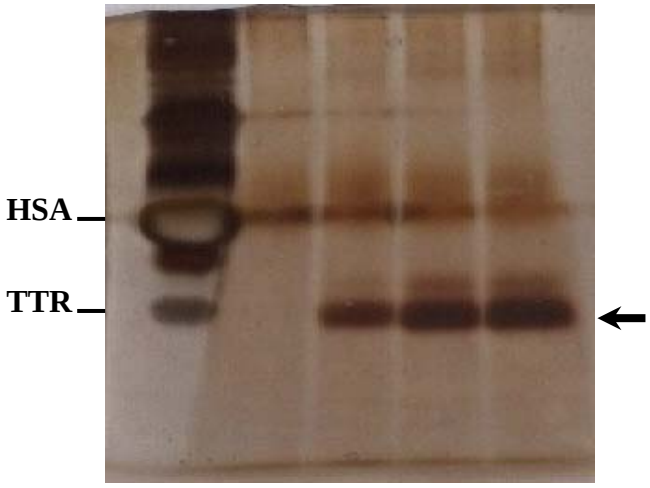
A



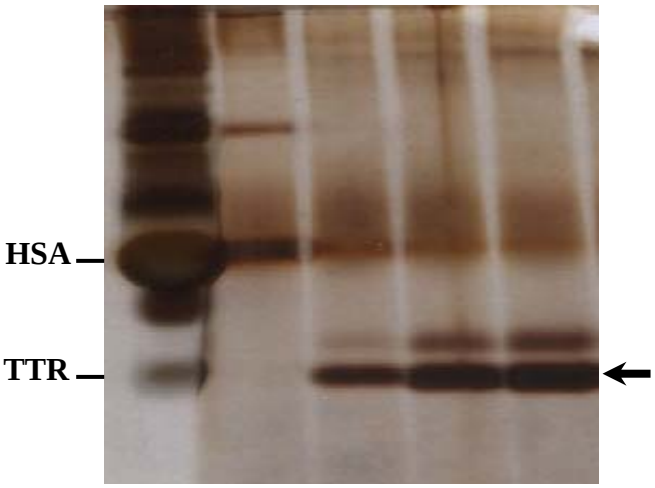
B



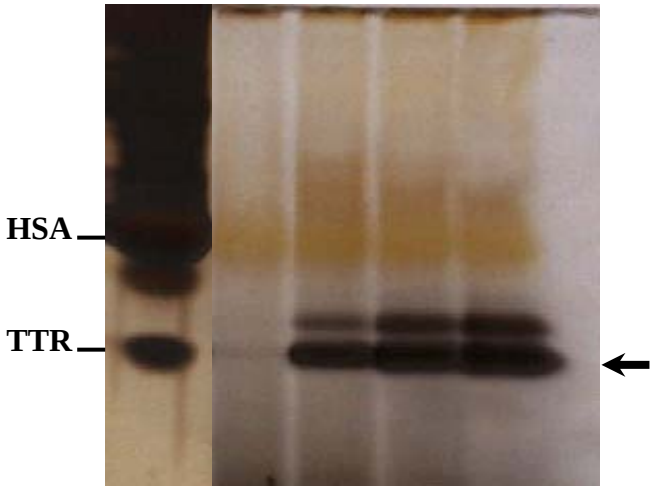
C



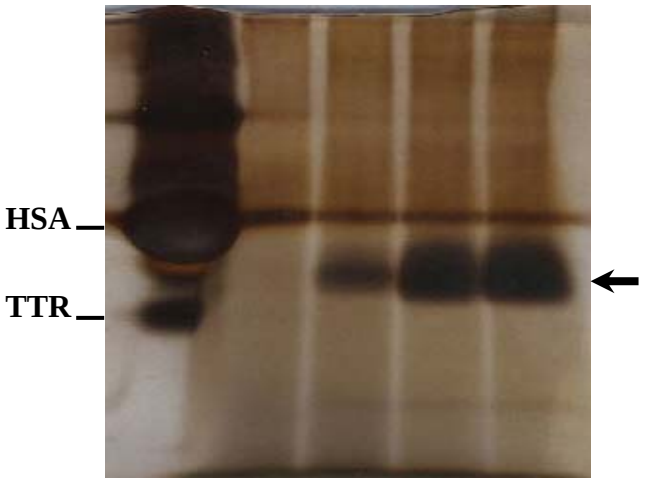
D



E



F



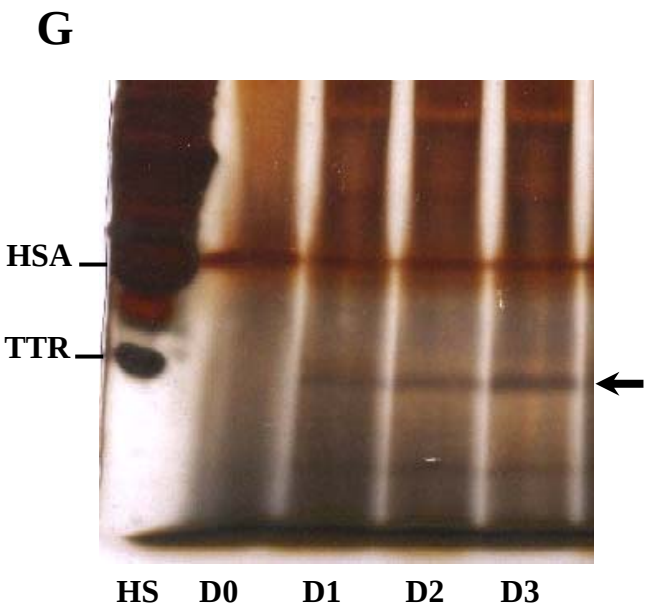


Figure 3.7 Kinetic expression of the recombinant TTRs in *P. pasrotis*

The *Pichia* recombinant clone was induced with methanol for protein synthesis in large scale for 3 to 4 days. The culture medium was collected every 24 h, and aliquot of the culture supernatant was analyzed for the protein production. HS is human serum, which was overloaded to show positions of albumin (HSA) and TTR. Position of the expected TTR band of each clone was indicated by arrow. D0 to D4 is day of the induction with methanol. (A), recombinant huTTR; (B), croc/huTTR; (C), crocTTR; (D), hu/crocTTR; (E), xeno/crocTTR; (F), pigC/crocTTR; (G), truncated crocTTR.

Table 3.1 The synthesis condition for large scale and the production yield of the recombinant TTRs

Recombinant TTR	Methanol concentration (%)	Induction period (days)	Yield of TTR synthesis (mg/L of culture)
huTTR	1	4	9
croc/huTTR	0.5	3	147
crocTTR	0.5	3	152
hu/crocTTR	0.5	3	220
xeno/crocTTR	0.5	3	147
pigC/crocTTR	0.5	3	60
truncated crocTTR	1	3	3

4. Purification of the recombinant TTRs

P. pastoris has a characteristic that make it becomes a more desirable host i.e. it secretes only small amount of the endogenous proteins. This facilitates purification of foreign proteins from other organisms. The recombinant TTRs could be purified from the culture medium by affinity chromatography on a human RBP-Sepharose 4B column or by preparative discontinuous native-PAGE using the Bio-Rad Prep Cell. A single step purification by RBP-Sepharose column was an efficient method to purify recombinant TTRs from yeast culture medium (Prapunpoj *et al*., 2000 and 2002) based on the fact that these two protein specifically bind to form a complex in plasma (Kanei *et al*., 1968 and Peterson, 1971). However, large amount of RBP is needed for preparing the column. Although commercial RBP is available, it is too expensive for preparing a preparative column. An alternative purification method is, thus, required. It has been demonstrated that TTR in plasma from all vertebrate species except those from pig and cattle migrated faster than albumin and other proteins in plasma during non-denaturing electrophoresis (Ingbar, 1958). This typical characteristic enabled to purify TTR by the preparative polyacrylamide gel electrophoresis under native condition (Richardson *et al*., 1994). The recombinant TTRs produced by *P. pastoris* could also be purified by this method. According to the kinetic secretion analysis, all

the recombinant TTRs synthesized by yeast in this thesis had similar electrophoretic mobilities to that observed in human TTR in plasma. Thus, the recombinant TTRs produced in this thesis were purified by the preparative native-PAGE. The additional purification on the RBP-Sepharose column was performed only when contamination by other proteins was still observed after the preparative native-PAGE step. In the preparative native-PAGE, the culture supernatant was concentrated prior it was applied onto a polyacrylamide gel column. The separation was carried out as described in the Materials and Methods section 2.3. The eluting fractions were collected and analyzed by native-PAGE (Figure 3.8). A single protein with migration corresponding to TTR was detected, indicating an effective of this separation method. The TTR fractions were pooled, concentrated and stored for further studies. The recombinant huTTR and truncated crocTTR could not be effectively purified by the preparative native-PAGE because of their small amount of the production. Large amount of other proteins co-secreted into the culture medium made it very difficult to isolate the TTRs. In contrast, by the affinity chromatography on the RBP-Sepharose column, the TTRs could be efficiently separated from the other contaminated proteins. Only a single protein was eluted out from the column (Figure 3.9). According to the specific interaction between TTR and RBP, the RBP-Sepharose column benefited not only for the purification but also for confirming the presence and correct property of the recombinant TTRs produced by *P. pastoris*. By the preparative native-PAGE, the purified recombinant TTRs was obtained in range 1 to 10 mg per 1 liter of the yeast culture. This was less than that previously reported for the native crocTTR, which provided up to 16 mg per 1 liter of yeast culture (Prapunpoj *et al*., 2002).

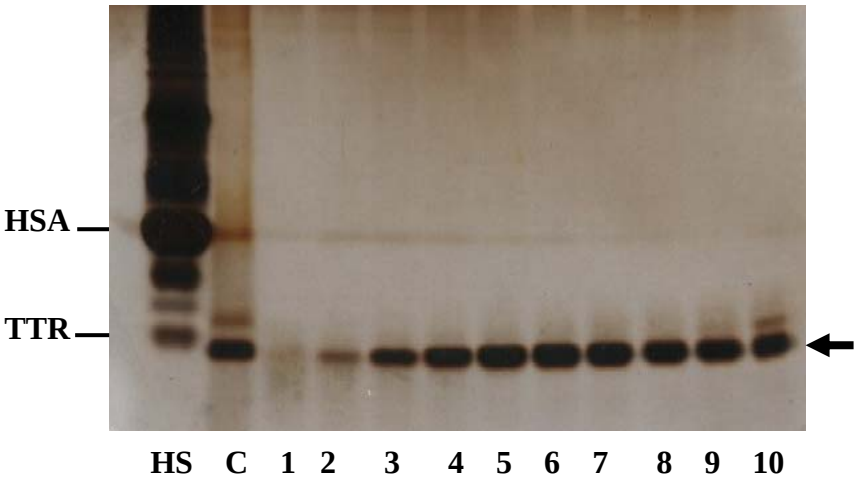


Figure 3.8 The elution pattern of TTR from the preparative native polyacrylamide gel

The recombinant TTRs could be separated from other proteins in culture medium by preparative native-PAGE. The concentrated culture supernatant was applied onto a cylindrical polyacrylamide gel column (12% resolving and 4%stacking gels; 55 ml of gel). Eluting fractions were collected (2 ml/fraction) at flow rate 60 ml/h. To determine the presence of TTR in fractions, an aliquot (10 µl) of each fraction was analyzed by native-PAGE (10% resolving and 4%stacking gels). The protein bands were detected by staining with silver nitrate. HS, human serum; C, concentrate culture supernatant; 1 to 10, an individual eluting fractions; HSA, albumin in human serum; TTR, TTR in human serum. The expected TTR band in each fraction was indicated by arrow.

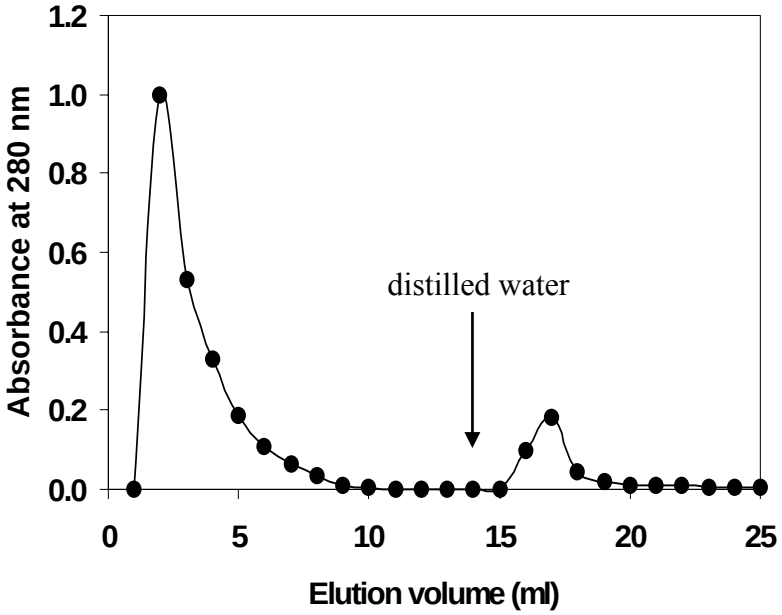


Figure 3.9 The affinity chromatographic pattern of the recombinant TTR on a human RBP-Sepharose 4B column

The induction synthesis for the recombinant huTTR was carried out with 0.5% methanol for 4 days. The culture supernatant was applied onto a column of human RBP-Sepharose 4B (5 ml of gel) equilibrated in 0.05 M Tris/HCl, pH7.4 buffer containing 0.5 M NaCl. The column was washed and the bound protein was eluted with distilled water as described in the Materials and Methods section2.3. The chromatographic separation was carried out at 4°C at flow rate 10 ml/h, and fractions were collected at 4 ml/fraction. The elution with distilled water was indicated by arrow.

5. Purification of human TTR from plasma

The TTR from human plasma was used as a control in the experiments conducted in this research. The protein was purified with two to three steps of purification. The first step was the purification by affinity chromatography on Cibacron blue column to remove an excess albumin in the pooled plasma. The second step was the separation of TTR from other contaminated proteins by preparative native-PAGE. In plasma, RBP always complexes with TTR, and dissociation of the complex could not occur simply. The separation out of bound RBP from TTR could be accomplished in the final step of purification by an electrophoresis on urea gel. In all TTR preparations that the contamination by RBP was observed, the purification by the electrophoresis on urea gel was carried on.

In the first step of purification by affinity chromatography on Cibacron blue column revealed that almost all of albumin, which was the major protein in blood, could be removed out with high salt (data not shown) from the pooled plasma (Figure 3.10A), though there were other proteins still contaminated. The TTR containing fractions were then pooled, concentrated and further purified by preparative native-PAGE (12% resolving and 4% stacking gels) in next step. The eluting fractions were collected and analyzed for TTR by native-PAGE as shown in Figure 3.10B. Only a single band that was corresponded to TTR was detected in each fraction. This demonstrated that TTR was completely isolated from the other proteins those appeared in the plasma. The fractions were pooled, concentrated prior the purity and the possible presence of RBP in fractions was determined by SDS-PAGE. Most of the preparative native-PAGE fractions of the human TTR produced in this thesis had no or very little contamination, determined by Coomassie blue staining, with RBP (Figure 3.10C). The purification step on urea gel was seldom performed.

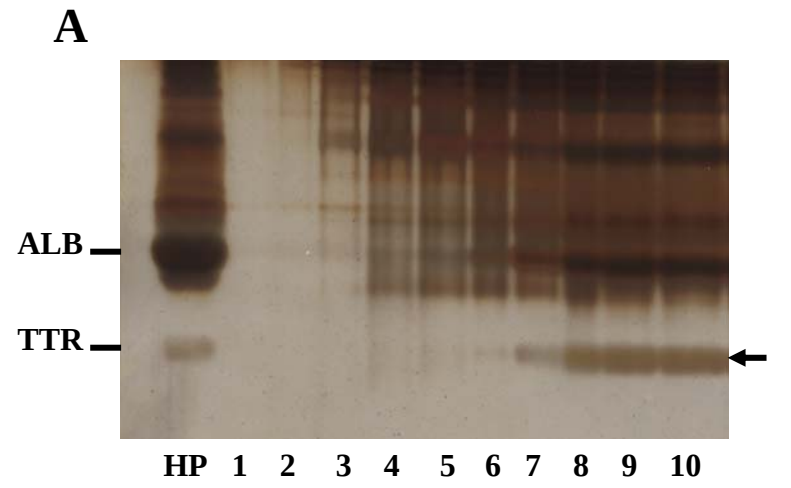

A
ALB
TTR
HP 1 2 3 4 5 6 7 8 9 10
B
ALB
TTR
HP 1 2 3 4 5 6 7 8 9 10
C
94
67
45
30
20.1
14.4
dimer
monomer
M
TTR

Figure 3.10 Purification of native TTR from human plasma

Pooled human plasma was purified by affinity chromatography on a Cibacron blue column. The unbound proteins were collected and analyzed by native-PAGE (A). The next step of purification was carried on by a preparative native PAGE. The fractions were collected and determined for TTR by native-PAGE (B). HP, pooled human plasma; 1 to 10, unbound fractions from the affinity chromatography step on Cibacron blue column or the fractions from the preparative native-PAGE. The protein bands corresponded to TTR were indicated by arrow. The purity of the TTR was then analyzed by SDS-PAGE (C). M is standard protein markers, the positions of TTR monomer and dimer were indicated.

6. Physicochemical properties of the recombinants TTRs

Although heterologous protein expression system of *P. pastoris* has a potential to perform many of post-translational modifications present in higher eukaryotes. Some of these slightly differ from those in mammals. For example, the carbohydrate moieties those incorporated into the secreted proteins produced by *P. pastoris* are predominantly composed of mannose residues. In addition, the hyperglycosylation was often detected in proteins (Cereghino and Cregg, 2000). These different post-translational modifications between *P. pastoris* and higher eukaryotes can lead to the alterations of properties and, as a consequence, to function of the recombinant proteins. To ascertain whether or not the unwanted post-translational modifications occurred to the recombinant TTRs synthesized in *P. pastoris*, physicochemical properties including the N-terminal sequence, electrophoresis mobility under non-denaturing condition, molecular weight of the tetramer, subunit molecular mass as well as immunochemical-reactivity of the recombinant TTR were studied.

6.1 Mobility in non-denaturing gel

During electrophoresis at pH 8.6, most TTRs from vertebrates including human (Seibert and Nelson, 1942) and birds (Chang *et al*., 1999) migrate faster than albumin. There are only some eutherian species such as pig and cattle that TTRs were found co-migrate with albumin in non-denaturing gel (Farer *et al*., 1962 and Refetoff *et al*., 1970). From the analysis, single band of a protein that corresponds to the TTR tetramer was found in all recombinant TTRs. The mobility of all recombinant TTRs were similar to that found in the human TTR purified from plasma. The exception was found in pigC/crocTTR and truncated crocTTR in which the proteins migrated slightly slower than other recombinant TTRs and the human TTR purified from plasma. The difference in the overall charges of the recombinant TTRs may be the reason of difference in the mobility. However, all TTRs migrated faster than albumin in human serum (Figure 3.11). This correlated to the typical characteristic of most vertebrate TTRs found in nature (Richardson *et al*., 1994). The results obtained implied that the recombinant TTRs produced by *P. pastoris* had the same proper physiological structure and molecular folding as those of the native TTRs.

6.2 Mobility in SDS-PAGE

In order to determine subunit molecular mass of the recombinant TTRs, the analysis by SDS-PAGE (12% resolving and 4% stacking gel) was adopted and protein bands were visualized by staining gel with Coomassie blue as described in the Materials and Methods section 2.4.2. The migration of TTRs was as shown in Figure 3.12. All recombinant TTRs showed slightly difference in mobility in comparing to each others. A major protein band that corresponded to TTR monomer was detected. An additional band, which corresponded to TTR dimer, migrated slower with much less intensity was also observed. The dimer of TTR usually occurs when denaturing of the protein is not complete even with hash condition (Dickson *et al*., 1982; Furuya *et al*., 1989 and Prapunpoj *et al*., 200b and 2002), indicating to strength of dimer-dimer interaction in the TTR molecule. The subunit masses of crocTTR, hu/crocTTR, xeno/crocTTR and pigC/crocTTR were 15.1, 15.5, 15.5 and 15.1 kDa, respectively. The truncated crocTTR showed a subunit mass of 15.5 kDa. Sometimes two addition discrete bands with masses of 16.6 kDa and 15.1 kDa could be observed. The subunit masses of the recombinant huTTR and croc/huTTR were 17.8 and 17.0 kDa, respectively. The subunit masses of the recombinant huTTR and its chimera produced by *P. pastoris* were similar to that of the human TTR purified from plasma (16.6 kDa). The subunit masses of the recombinant TTRs calculated directly from the deduced amino acid were 14.4, 14.8, 13.2, 13.7, 14.1, 13.7 and 14.1 kDa for crocTTR, hu/crocTTR, xeno/crocTTR, pigC/crocTTR, truncated crocTTR, huTTR and croc/huTTR, respectively (Table 3.2). In comparison, the subunit masses determined by SDS-PAGE were not much different from those directly determined from the derived amino acid sequences.

6.3 Molecular weight of the recombinant TTR tetramers

Molecular weights of all the recombinant TTRs synthesized in *P. pastoris* were determined by HPLC using the Bio Sil-SEC column. The column was calibrated as described in the Materials and Methods section 2.4.1. The results revealed that the tetrameric masses of crocTTR, hu/crocTTR, xeno/crocTTR, pigC/crocTTR and truncated crocTTR were 60, 63, 66, 54 and 51 kDa, respectively, whereas both huTTR and hu/crocN TTR had the same molecular weight i.e. 63 kDa (Figure 3.13),

similar to that observed in the TTR purified from human plasma. The molecular weights of the recombinant TTRs were in range of those reported for TTR from many vertebrate species, so it revealed the tetramers of the recombinant TTRs could be successfully formed in *P. pastoris*.

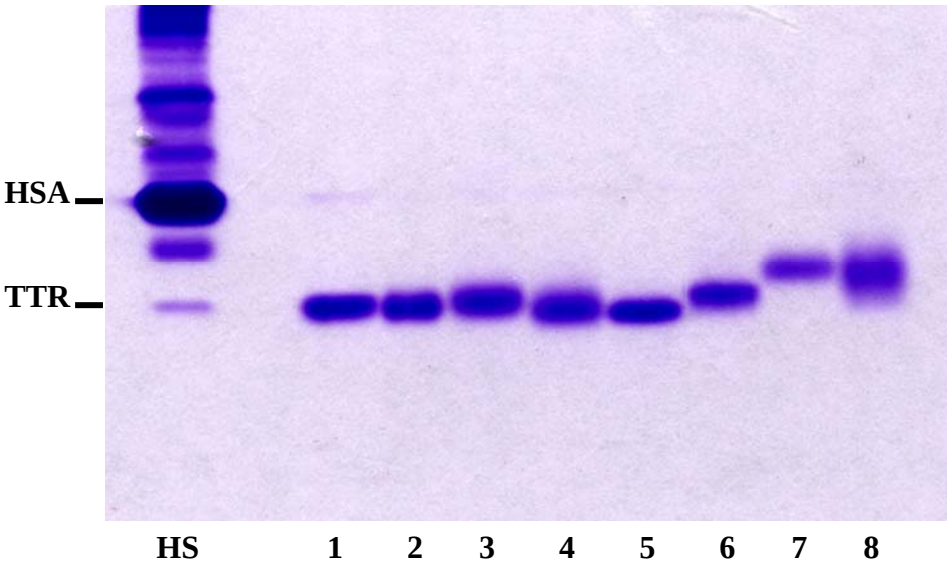
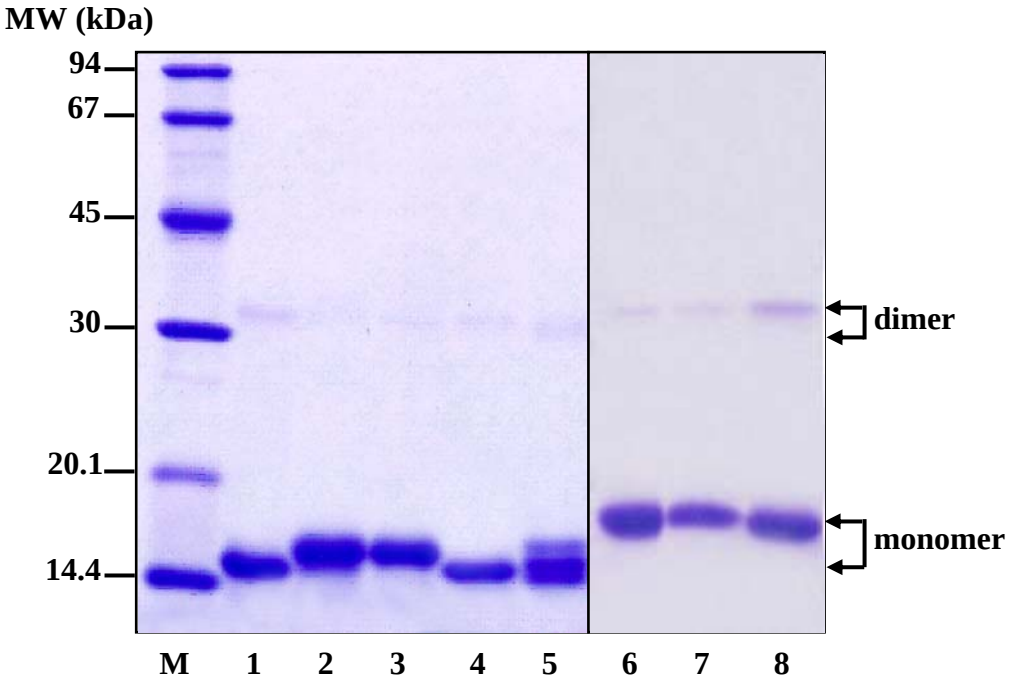


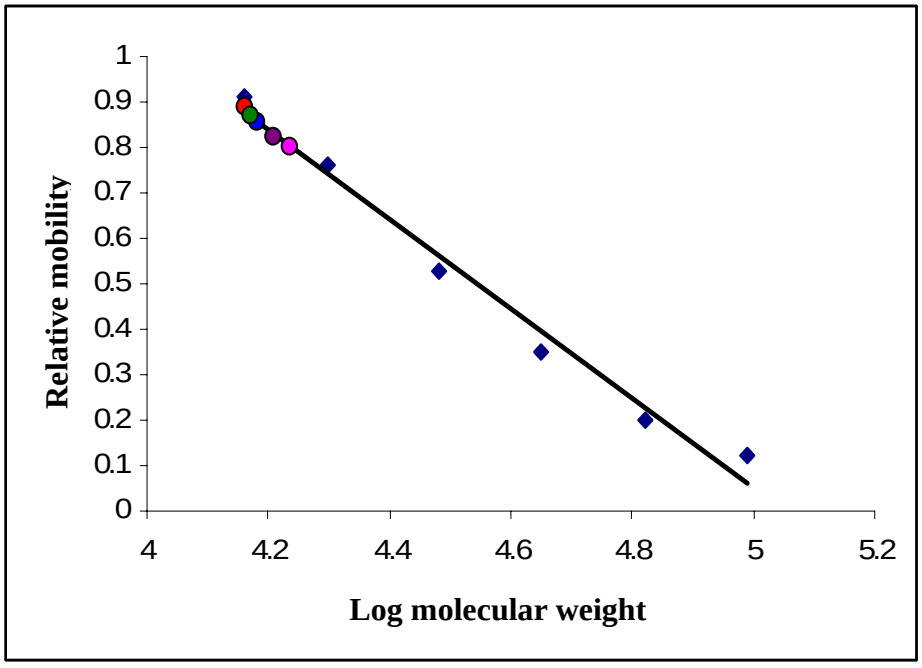
Figure 3.11 The electrophoretic mobility under native condition of the recombinant TTRs

Two micrograms of the purified human TTR from plasma (1), recombinant huTTR (2), croc/huTTR (3), crocTTR (4), hu/crocTTR (5), xe/crocTTR (6), pigC/crocTTR (7) and truncated crocTTR (8) were separated under non-denaturing condition on PAGE (10% resolving and 4% stacking gels). Protein bands were visualized by Coomassie blue staining. Human serum (HS) was overloaded to indicate the TTR (TTR) and albumin (HSA) in human serum.

A



B



crocTTR, pigC/crocTTR, truncated crocTTR band 3

hu/crocTTR, xeno/crocTTR, truncatd crocTTR band 2

truncated crocTTR band 1

Recombinant huTTR, plasma human TTR

croc/huTTR

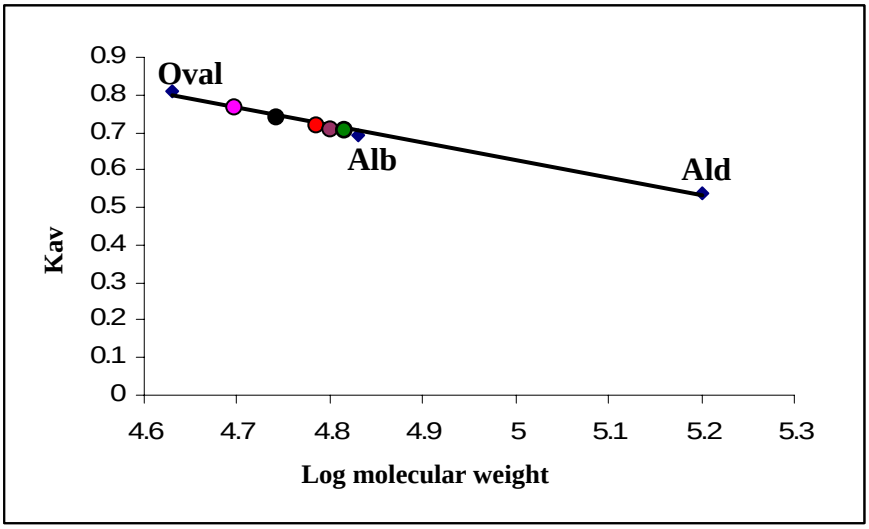
Figure 3.12 Analysis by SDS-PAGE and determination of the subunit masses of the recombinant TTRs

Two micrograms of the purified recombinant crocTTR (1), hu/crocTTR (2), xeno/crocTTR (3), pigC/crocTTR (4), truncated crocTTR (5), plasma huTTR (6), recombinant huTTR (7) and croc/huTTR (8) were boiled for 30 min in the presence of 2.5% 2-mercaptoethanol and 2% SDS prior the protein separation was carried out by 12% SDS-PAGE (A). Protein bands were stained with Coomassie blue. M was standard protein markers. Positions of TTR monomer and dimer were indicated. The TTR subunit masses were calculated from the standard curve plotted between the relative mobility (R_f) and the logarithmic value of the protein marker masses (B). The relative mobilities of all TTRs also were shown.

Table 3.2 Subunit masses of recombinant TTRs determined by SDS-PAGE and those directly calculated from the deduced amino acid sequences

TTR	Subunit mass by SDS-PAGE (kDa)	Subunit mass from deduced amino acid sequence (kDa)
huTTR	17.8	13.7
croc/huTTR	17.0	14.1
crocTTR	15.1	14.4
hu/crocTTR	15.5	14.8
xeno/crocTTR	15.5	13.2
pigC/crocTTR	15.1	13.7
truncated crocTTR band1	16.6	14.1
truncated crocTTR band2	15.5 (main subunit)	
truncated crocTTR band3	15.1	

A



B

	Molecular weight (kDa)
huTTR	63
croc/huTTR	63
crocTTR	60
hu/crocTTR	63
xeno/crocTTR	66
pigC/crocTTR	54
truncated crocTTR	51

Figure 3.13 Molecular mass determination of the recombinant TTRs.

The masses of TTR tetramer were analyzed by HPLC using a Bio-Sil SEC column as mentioned in the Materials and Methods section 2.4.1. The standard protein markers included aldorase (Ald) (158 kDa), albumin (Alb) (67 kDa) and Ovalbumin (Oval)(43 kDa). The Kav of all the recombinant TTRs were indicated in (A). The molecular weights were summarized (B).

6.4 Immunochemical reactivity with antibodies against TTR

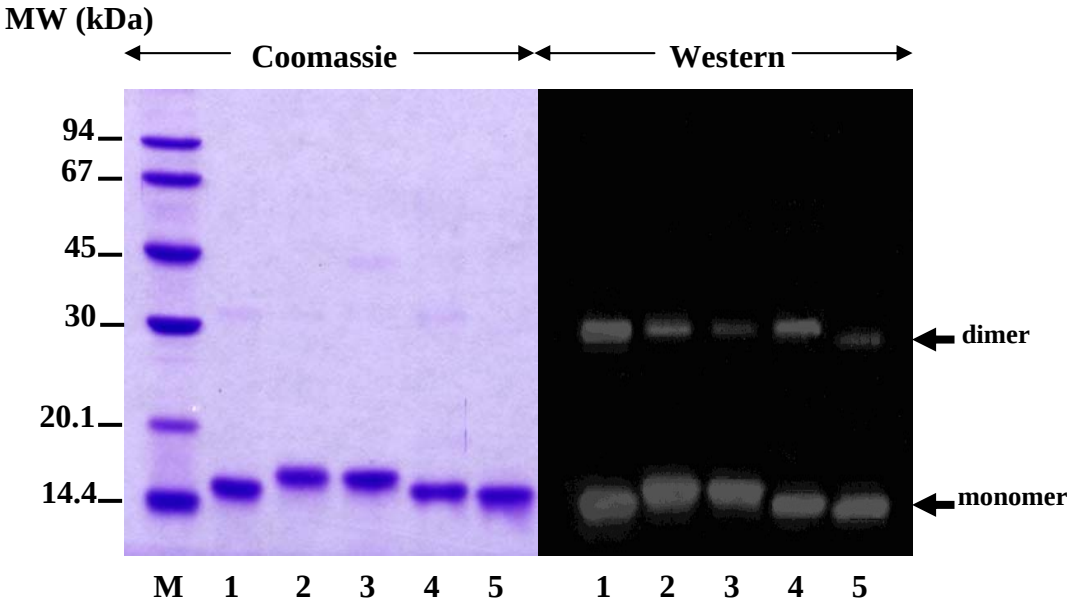
The immunochemical reactivity to a specific antibody is one of the effective strategies to identify or confirm the presence of the protein of interest. The recombinant crocTTR was demonstrated having the cross-reactivity to the antibody raised against a mixture of human TTR purified from serum, wallaby TTR and chicken TTR (Prapupoj *et al*., 2002). In this thesis, a polyclonal antibody that specific to the recombinant crocTTR was produced as described in the Materials and Methods section 2.16. Together with the available specific antibody to human TTR (Abcam) and that to the mixture of serum human TTR, wallaby TTR and chicken TTR (Richardson *et al*., 1996), the crocTTR antibody was used to determine the cross-reactivity of the recombinant TTRs synthesized by *P. pastoris* and used in all experiments. The purified TTRs were resolved on SDS-PAGE prior they were electrophoretically transferred to nitrocellulose membranes. The antiserum against human TTR purified from serum or against a mixture of TTRs or against recombinant crocTTR was used as a primary antibody as described in the Materials and Methods section 2.6 and Table 2.2. After the peroxidase activity was developed and the signal was detected by ECL, two bands with immuno-reactivity signal were observed for all recombinant TTRs (Figure 3.14). The first band, with very high intensity, migrated to the same position corresponded to the TTR subunit that was detected by staining with Coomassie blue. The other band migrated slower with a mass ~30 kDa, corresponding to the TTR dimer (Figure 3.14A and 3.14B). These results confirmed the identity of all recombinant TTRs.

6.5 The N-teminal sequencing

In order to ascertain whether the recombinant native and chimeric TTRs were produced with corrected amino acid particular at the N-termini, the N-terminal sequence of the proteins were carried out. It revealed that the first four or eight amino acids in the N-terminal sequences of the recombinant huTTR, crocTTR xeno/crocTTR and truncated crocTTR were **G P T G** (from this thesis and Prapunpoj *et al*., 2006), **A P L V S H G S** (Prapunpoj *et al*., 2002), **A P P G H A S H** (Prapunpoj *et al*., 2002) and **S K C P** (from this thesis), respectively. These well agreed with the expected N-terminal sequences of the TTRs. However, two addition

amino acid residues, E and A, were detected at the N-terminal sequence of hu/crocTTR led to **E A G P** as the first four amino acid at the hu/crocTTR N-terminus (from this thesis and Prapunpoj *et al*., 2006).

A


MW (kDa)
Coomassie
Western
94
67
45
30
20.1
14.4
dimer
monomer
M 1 2 3 4 5 1 2 3 4 5

B

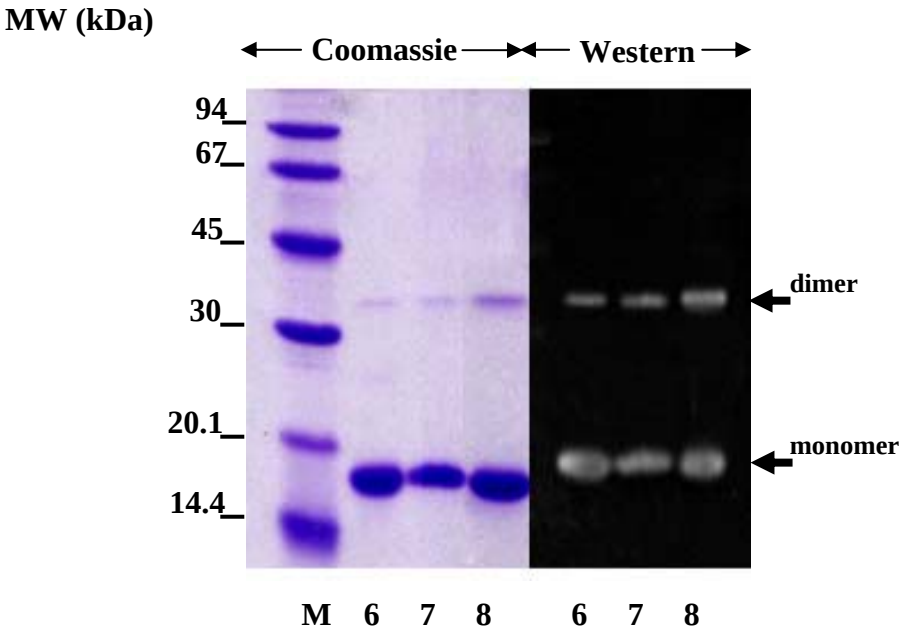

MW (kDa)
Coomassie
Western
94
67
45
30
20.1
14.4
dimer
monomer
M 6 7 8 6 7 8

Figure 3.14 Western analysis of the recombinant TTRs

The recombinant TTRs were subjected to Western analysis as described in section 2.6. The protein band were stained with Coomassie blue (Coomassie) while Western analysis (western) was carried out using rabbit antiserum raised against crocTTR (1:500) (A) or sheep antiserum against serum huTTR (1:2500) (B) as the primary antibody. The horseradish peroxidase (HRP)-linked rabbit IgG antibody and the HRP-linked sheep IgG antibody at 1:2500 dilution were used as the secondary antibody for crocTTR and serum huTTR, respectively. M, standard low molecular weight protein markers; 1, crocTTR; 2, hu/crocTTR; 3, xeno/crocTTR; 4, pigC/crocTTR; 5, truncated crocTTR; 6, plasma huTTR; 7, recombinant huTTR; 8, croc/huTTR. Detection was performed using Enhance Chemiluminescence kit (Amersham). The position of TTR monomer and dimer were indicated.

PART II: INFLUENCE OF THE N- AND C-TERMINAL REGIONS ON FUNCTIONS OF TTRS

7. Influence of the N-terminal region on the binding of TTR to THs

One important function of TTR is to ensure the appropriate extracellular and intracellular distribution of THs (for review see Schreiber and Richardson, 1997). Multiple amino acid sequence alignments of TTR monomer from several vertebrate species show a high degree of conservation for this protein. Moreover, the amino acids that involved in the central channel of the binding sites for THs are almost 100% conserved during evolution. This can suggest to the important function as a THs carrier of TTR. The predominant change in amino acid sequence occurred at the N-terminus of TTR. This segment is longer and more hydrophobic in TTRs from amphibian, reptilian, bird (with three additional amino acids) and in some marsupials (with two additional amino acids) than those from eutherians. The splice site shift of intron1 has been proposed to be a molecular mechanism underlying of this alteration (Aldred *et al*., 1997). The co-incident evidence showed that the affinities for T_3 and T_4 of TTRs from vertebrates unidirectional varied during evolution (Chang *et al*., 1999). The TTRs from fish (Santos *et al*., 1999 and Mazon *et al*., 2007), amphibians (Yamauchi *et al*., 1993 and Prapunpoj *et al*., 2000b), reptiles (Prapunpoj *et al*., 2002) and birds (Chang *et al*., 1999) bind T_3 better than T_4, whereas TTRs from eutherians include human bind T_4 with higher affinity than T_3. Together with the fact that the N-terminal region located at the entrance to the channel where the binding sites for THs exist, the question arises whether length and hydropathy of the N-terminus has influence on the binding to THs and other functions of TTR. To elucidate, the synthesis of chimeric TTRs in which the N-terminal region is altered has been shown to be a useful tool. In previous study, the influence of the N-terminal region on the binding affinities of TTR to T_3 and T_4 was demonstrated using wild type *C. porosus* TTR and one of its chimera in which the N-terminal sequence of the crocodile TTR was changed to that of *X. laevis* TTR as models (Prapunpoj *et al*., 2002). However, both *C. porosus* and *X. laevis* TTRs prefer binding to T_3 than T_4 (Prapunpoj *et al*., 2000b, 2002), and the experiment via using TTRs with different TH binding preference has not been performed yet. To gain more insight of the influence of the N-

terminal region, the affinities for T_3 and T_4 were elucidated using the chimeric TTRs those constructed to contain the N-terminal segments from the other TTR with different TH binding preferences or truncated. These included the hu/crocTTR, which was a chimeric of *C. porosus* TTR (prefers to bind T_3) that contained the N-terminal sequence of human TTR (prefers to bind T_4), and the truncated *C. porosus* TTR (truncated crocTTR), which was a chimeric crocodile TTR in which the first 12 amino acid residues at the N-terminus were removed out. The affinities to T_3 and T_4 of these chimeras were calculated and compared to those wild type *C. porosus* and human TTRs.

The dissociation constant (K_d) that quantitatively characterized the binding of two molecules was used to revealed the binding of TTR to THs in this thesis. The K_d values for binding to TH of TTR varied depending on the method used such as the binding of human TTR to T_4 varied from 0.3 to 128 nM (Chang *et al*., 1999). In this thesis the binding affinity of TTRs to T_4 and T_3 were determined using a high reproducible, rapid and sensitive method developed by Chang *et al*., 1999. To minimizing undesirable surface contact of THs, which always resulted in non-specific binding, this method is incorporated with several corrections. These include using the methyl cellulose coated activated charcoal to separate out the TTR-TH complex from free TH and the bound charcoal is completely separated out from the equilibration mixture within 3 s by vacuum. In addition, non-specific binding is extrapolated with corrections. In this thesis, three individual experiments were performed for each hormone. The binding curves were plotted following the general equation according to Scatchard, 1949:

$$r/[u] = K_a n - K_a r = -1/K_d[r-n]$$

where r is the fraction of available binding sites on a macromolecule, which is occupied by a ligand, [u] is the free ligand concentration, n is the number of binding sites per macromolecule, K_a is the intrinsic association constant, and K_d is the dissociation constant.

For analysis, least square of linear regression line was calculated with 95% confidence using the statistical package in Microsoft Excel, and the K_d values were

derived from their slopes. The binding capacity to THs was derived from intercept with the abscissa in the Scatchard plot.

The K_d values derived from the Scatchard analysis and plots of the recombinant huTTR were 53.26 ± 3.97 nM for T_3 and 19.73 ± 0.13 nM for T_4 leading to the Kd T_3/Kd T_4 ratio to 2.70 (Figure 3.15A, B; Table 3.3). This was similar to those reported for the TTR purified from human serum (Chang *et al*., 1999). The binding capacities for both T_3 and T_4 of the huTTR, which were derived from the abscissa interception of the binding curves, suggested a capacity of two molecules of THs per TTR molecule.

Compared with wild type *C. porosus* TTR, the recombinant chimeric TTRs, i.e. hu/crocTTR and truncated crocTTR, had different binding affinities to T_3 and T_4 and the K_d T_3/K_d T_4 ratios. The K_d for T_3 and T_4 of the hu/crocTTR were 5.40 ± 0.25 nM and 22.75 ±1.89 nM, respectively, providing a K_d T_3/K_d T_4 ratio of 0.24 (Figure 3.15C, D and Table 3.3). This K_d ratio was higher than that previous reported for *C. porosus* TTR (Prapunpoj *et al*., 2002). Since the K_d for T_3 of hu/crocTTR was not significantly different from that of *C. porosus* TTR (7.56 ± 0.84 nM), the higher K_d T_4/K_d T_3 ratio could indicate greater influence of changes in the N-terminal sequence on binding of the TTR to T_4 than to T_3. In contrast, the K_d for both T_3 and T_4 of the truncated crocTTR were similar. The truncated crocTTR bound to T_3 with the K_d of 57.58 ± 5.65 nM and to T_4 with the K_d of 59.72 ± 3.38 nM (Figure3.15E, F and Table 3.3) leading to a K_d T_3/K_d T_4 ratio of 0.97. The K_d values and the Kd ratios of the recombinant TTRs were summarized in Table3.3.

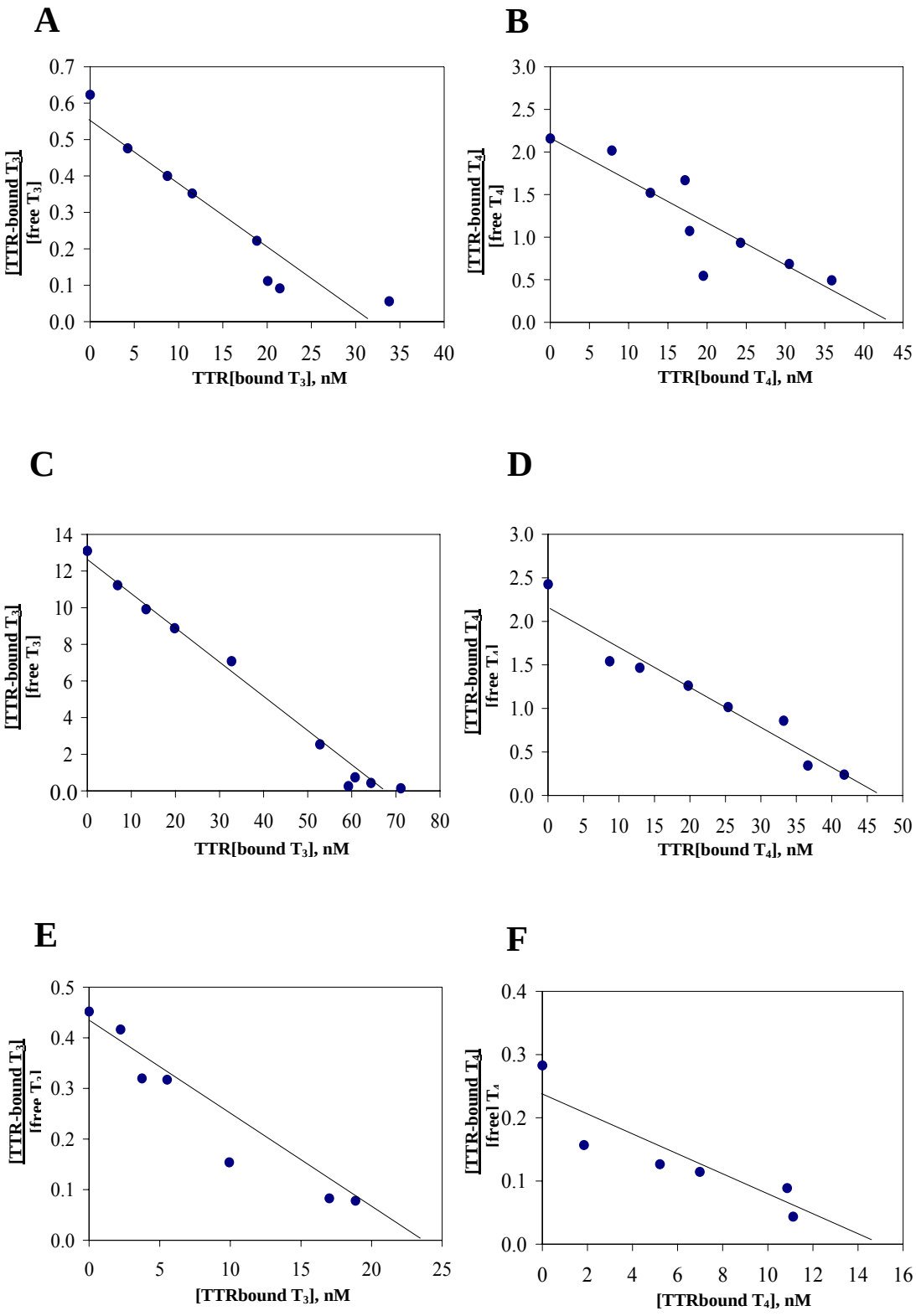

A
B
C
D
E
F
[TTR-bound T3] / [free T3]
[TTR-bound T4] / [free T4]
TTR[bound T3], nM
TTR[bound T4], nM
[TTRbound T3], nM
[TTRbound T4], nM

Figure 3.15 The binding of the recombinant TTRs to THs.

Purified recombinant TTRs (huTTR, A and B; hu/crocTTR, C and D; truncated crocTTR, E and F) (100 nM) were incubated with T_3 or T_4 in the presence of trace amount of $[^{125}I]$-T_3 or $[^{125}I]$-T_4 at 4°C, overnight. Free hormone was separated from the TTR bound hormone by filtering the incubation mixture through a layer of methyl cellulose-coated charcoal under vacuum as described in the Materials and Methods section 2.8. All corrections including those for non-specific binding were applied before performing Scatchard analysis.

Table 3.3 The binding dissociation constants (K_d) for T_3 and T_4 of the recombinant TTRs

TTR	K_d for T_3 (nM) *	K_d for T_4 (nM) *	K_dT_3/K_dT_4	Reference
recombinant huTTR	53.26±3.97	19.73±0.13	2.70	This thesis
hu/crocTTR	5.40±0.25	22.75±1.89	0.24	This thesis
truncated crocTTR	57.78±5.65	59.72±3.38	0.97	This thesis
crocTTR	7.56±0.84	36.73±2.38	0.21	Prapunpoj *et al*., 2002

* Results are presented as mean ± SD of the K_d (from 3 replicates)

8. Influence of N- and C-terminal regions on the binding of TTR to human RBP

One of the other main functions of TTR is to transport of vitamin A via binding to RBP, a single polypeptide with the mass of 21 kDa. The binding of TTR to RBP has believed to prevent loss of RBP through glomerular filtration in kidney (Kanai *et al*., 1968; Raz and Goodman, 1969; Goodman, 1974 and Noy *et al*., 1992). In similar to TTR, RBP is one of the most strongly conserved proteins in plasma. Conservation of the amino acid sequence in RBP during evolution was demonstrated (Berni *et al*., 1992). Interaction between TTR and RBP from different species that quite far distance in evolution were reported, including the complex between human TTR and chicken RBP (Kopelman *et al*., 1976), and that between human TTR and trout RBP (Berni *et al*., 1992) (for review see Monaco, 2000). Binding affinity of these two proteins varied, in both homologous (from same species) complex and chimeric (cross species) from 10^{-6}-10^{-7} M depending on the techniques used (Noy *et al*., 1992 and Kopelman *et al*., 1976). Stoichiometry of the binding revealed one RBP per TTR tetramer in *vivo*, and up to two RBP (for review sees Monoco, 2000). Both TTR and RBP contribute each 21 amino acids to the interaction of the complex, and most of these involved residues locate at C-terminal regions of the proteins (Naylor and Newcomer, 1999). There was no report mentioned to the involvement or influence of the TTR N-terminus on this interaction. However, since this region is remarkably change during evolution of vertebrates and locates closely to the C-terminal region at the entrance to the central channel of the TTR tetramer, the question arises whether changes in amino acid sequence of the N-terminal region involves with the binding between TTR and RBP. In order to elucidate, the binding interaction of the chimeric TTRs were performed and compared to the wild type TTRs. Determination of the binding interaction between TTR and RBP could be carried out by several techniques such as ultracentrifugation (Pieter *et al*., 1973), gel filtration (Heller and Horwitz, 1974), fluorescence polarization (Kopelman *et al*., 1976) and mass spectrometry (Rostom *et al*., 1998). In this thesis, the free and bound forms of TTR and RBP were separated by electrophoresis and quantitatively analyzed by western analysis using a specific antiserum as described in the Materials and Methods section 2.6. The method was chosen with several reasons, e.g. it is rapid and easy to perform, only small amount of TTR and RBP is required for an assay, and kinetic of the binding could be

directly observed from gel staining with Coomassie blue. In an assay, TTR and human RBP in different molar ratios were allowed to bind at 4°C for 1 h. Free TTR and RBP were separated from the TTR-RBP complex by native-PAGE prior the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane blotting was carried at a constant low voltage (25 volts; ~48-57 mA) for 30-40 min with cooling. The amounts of bound and free TTR were determined using two appropriate antibodies as described in the Methods and Materials section 2.6 and Table 2.2. To confirm the specific reactivity of the antibody, a possible cross-reactivity of the antibody with TTR and RBP were examined. The result showed that only TTR, but not RBP could interact with the antibody raised against TTR (Figure 3.16).

The kinetic of the binding between TTR and RBP could be directly observed in gel (Figure 3.17A). In comparison, the decreases of free hu/crocTTR and croc/huTTR in the presence of higher amount of RBP were similar to that of wild type human TTR purified from plasma, but slower than those of other TTRs. These implied to less binding affinities to human RBP of hu/crocTTR, croc/huTTR and wild type huTTR than the other TTRs.

These results were confirmed by the specific antibodies to TTR (Figure 3.17B). TTRs could be classified into 3 groups based on the binding rate. TTRs in the first group, including *C. porosus* TTR and all its chimeras except hu/crocTTR, had a rapid binding rate. The binding could first detected in the reaction mixture contained RBP:TTR ratio at 0.5:1, and all TTR was bound when RBP was increased to 4 folds. A chimeric TTR, i.e. hu/crocTTR, was in the second group that had intermediate rate of the binding. The binding was first detected at RBP:TTR molar ratio of 2:1, and was not completed even RBP was added to 8 folds. In the third group TTRs, including human TTR purified from plasma and croc/huTTR, had the slowest rate of the binding. The first detection of the binding occurred at RBP:TTR ratio of 2:1 to 4:1. Less binding still was observed even the concentration of RBP was raised to 8 folds, indicating very low initiation step of binding between this TTR and RBP. For croc/huTTR, the complex was first clearly observed when RBP:TTR was 12:1, and the binding almost complete at the ratio 32:1 (data not shown).

Although kinetic of the binding could be revealed in gel, the binding affinity could not be determined because of the overlapping between free RBP and the TTR-RBP complex. To determine a K_d by Scatchard analysis, two parameters, the concentration of bound and free RBP, are required in the equation. Although free and bound RBP could be detected by using a specific antibody raised against RBP, their amount could not be determined because of the overlapping of these two protein bands. In contrast, the protein bands of free and bound TTRs could be clearly separated from each other (Figure 3.17B), and the fluorescence signals of these two bands could be accurately determined. On the assumption that TTR binds to RBP at 1:1 molar ratio, the concentration of bound RBP could be directly determine from bound TTR and used to determine K_d. Free RBP could be directly obtained by subtraction of bound from total RBP. According to the electrophoretically blot of protein, the variable and less than 100% efficiency always obtained. To correct, so that the K_d from each experiment could be compared, the efficiency of transfer was calculated from the TTR control in the gel and this value was included in finding out the concentration of bound RBP.

From the Scatchard analysis, TTRs could be classified into three groups according to the K_d values. The recombinant wild type crocTTR and its chimeras except hu/crocTTR showed K_d with small values. These were 2.54±0.23 μM, 2.34±0.45 μM, 1.66±0.27 μM and 1.19±0.19 μM for crocTTR, pigC/crocTTR, xeno/crocTTR and truncated crocTTR, respectively (Figure 3.18, Table 3.4). The hu/crocTTR had a K_d for binding to RBP of 11.31±0.07 μM and was classified into the intermediate group. The native human TTR purified from plasma and its chimera, croc/huTTR, showed high K_d and these were classified in the third group. The K_d for RBP of these two proteins were 17.08±3.25 μM and 22.52±5.84 μM for human TTR and croc/huTTR, respectively. All K_d for RBP of TTRs obtained were in range of that reported (10^{-6}-10^{-7} μM) using other determination methods (Noy *et al.*, 1992) suggesting similar sensitivity of this to the other methods.

By comparing the K_d, crocTTR had higher affinity to human RBP than human TTR. In addition, the K_d for binding to RBP of crocTTR and all its chimeras were significantly different from that of human TTR and its chimera. Among the chimeric TTRs originated from crocTTR, hu/crocTTR showed significant change in K_d and this was 5 folds higher than that of the wild type crocTTR. This clearly demonstrated the

influence of the N-terminal sequence on binding of the TTR to RBP. However, alteration of the binding affinity to RBP was not observed in other chimeric crocTTRs and croc/huTTR in which the amino acid sequence was vice versa to that of hu/crocTTR. K_d of these TTRs was not significantly different from that of wild type crocTTR or human TTR (Table 3.4). Together with the hydropathy consideration, these results primary suggested that changing the N-terminal sequence to more hydrophilic (in case of hu/crocTTR) would decrease the affinity of TTR to RBP. In contrast, changing the N-terminal sequence to more hydrophobic (in case of croc/huTTR and other chimeric TTRs) would not alter the binding affinity. Although most of the amino acids from TTR that are involved in the TTR-RBP complex formation locate at the C-terminal region of the TTR subunit (Naylor and Newcomer, 1999), alteration in K_d of the pigC/crocTTR was not observed (Table 3.4). The binding affinity of the chimeric TTR was similar to that of wild type crocTTR. This should also imply to more important of amino acid residues in the core structure than those in the N- or C-terminal region to the binding and complex formation between TTR and RBP.

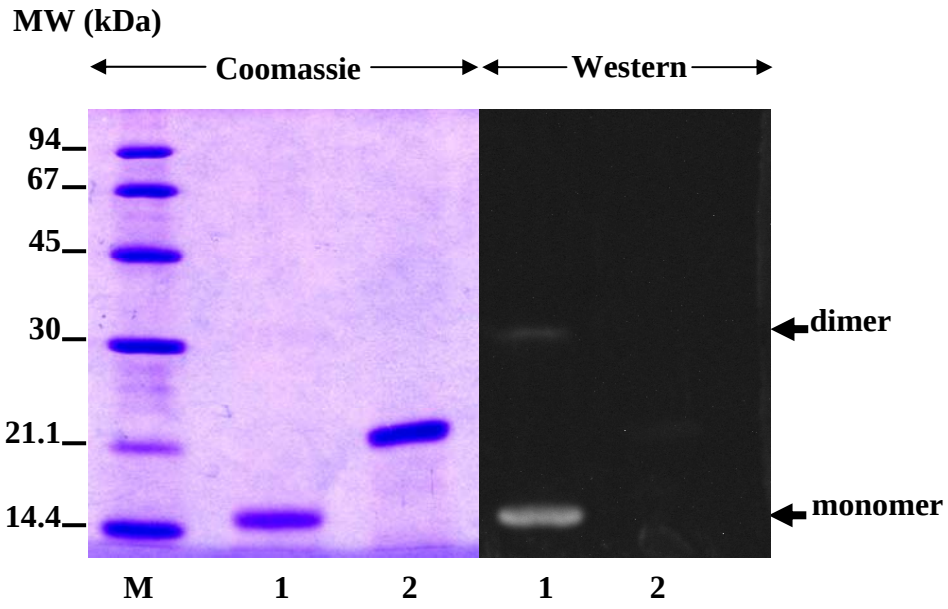
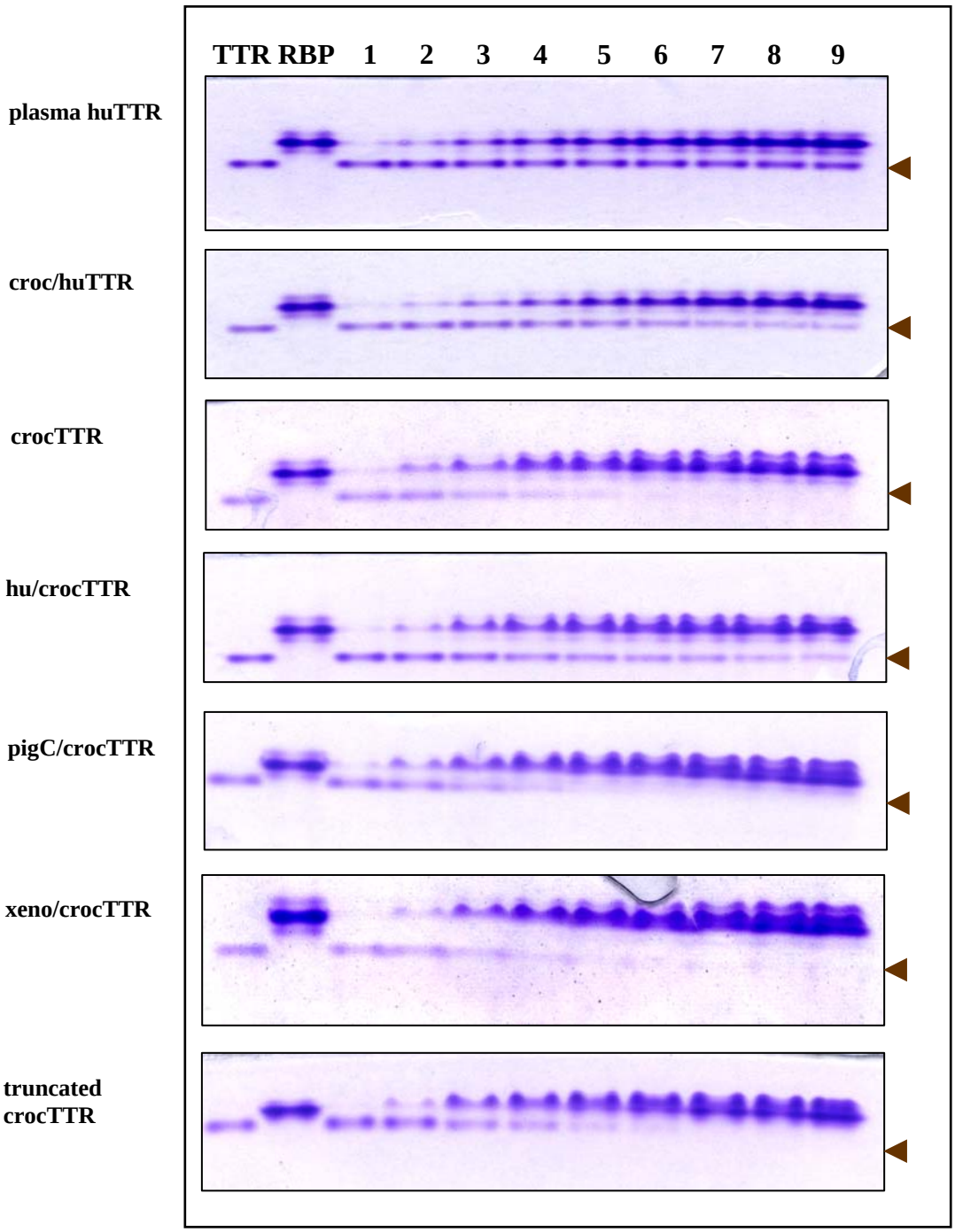


Figure 3.16 Analysis of cross-reactivity of hRBP to antiserum of TTR

One microgram of purified recombinant crocTTR (1) and RBP (2) were separated by SDS-PAGE (12% resolving and 4% stacking gels). The proteins were identified either by staining with Coomassie blue (Coomassie) or by reaction with antiserum (Western). The primary and the secondary used were the antiserum raised against crocTTR (1:500) and the anti-rabbit IgG antibody-HRP (1:2500), respectively. M is the standard protein markers. Positions of the TTR monomer and dimer were indicated.

A


TTR RBP 1 2 3 4 5 6 7 8 9
plasma huTTR
croc/huTTR
crocTTR
hu/crocTTR
pigC/crocTTR
xeno/crocTTR
truncated crocTTR

B

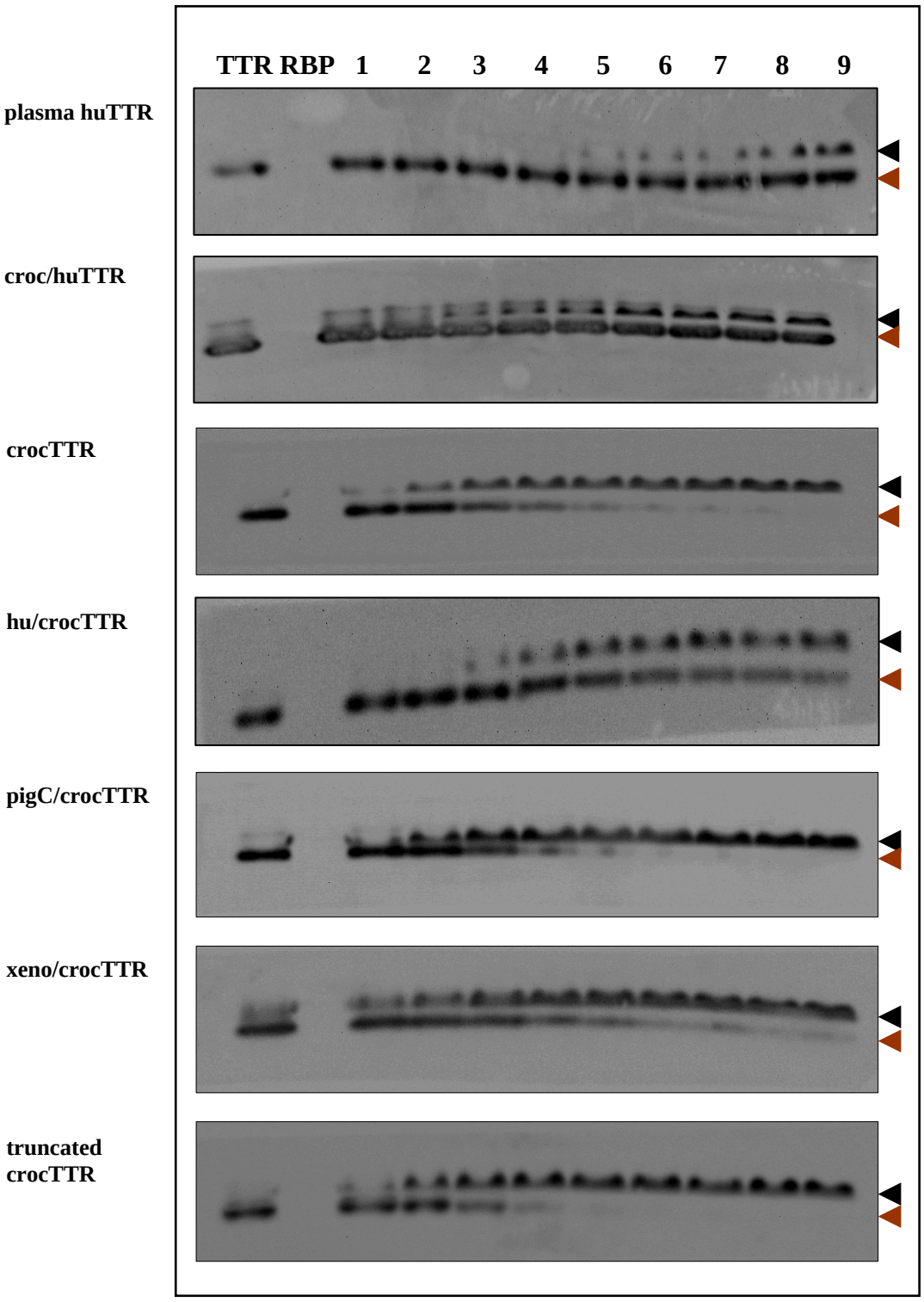

TTR RBP 1 2 3 4 5 6 7 8 9
plasma huTTR
croc/huTTR
crocTTR
hu/crocTTR
pigC/crocTTR
xeno/crocTTR
truncated crocTTR

Figure 3.17 Analysis of the binding of TTRs to human RBP

Purified recombinant TTR (0.4 μg) was incubated with various amount of purified human RBP (0 to 4 μM), at 4°C for 1 h. The reaction mixture was analyzed by native-PAGE (10% resolving and 4% stacking gels). Proteins were identified by staining with Coomassie blue (A). Bound and free TTRs were identified by western blot analysis followed by ECL using anti-human TTR or anti-crocTTR antibody as the primary antibody (B). Purified TTR (TTR) or human RBP (RBP) alone was added as controls. 1 to 9 was the reaction mixture contained RBP:TTR to a final molar ratio of 0.5:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 and 8:1, respectively. The bound and free TTRs were indicated by black and brown arrowhead, respectively.

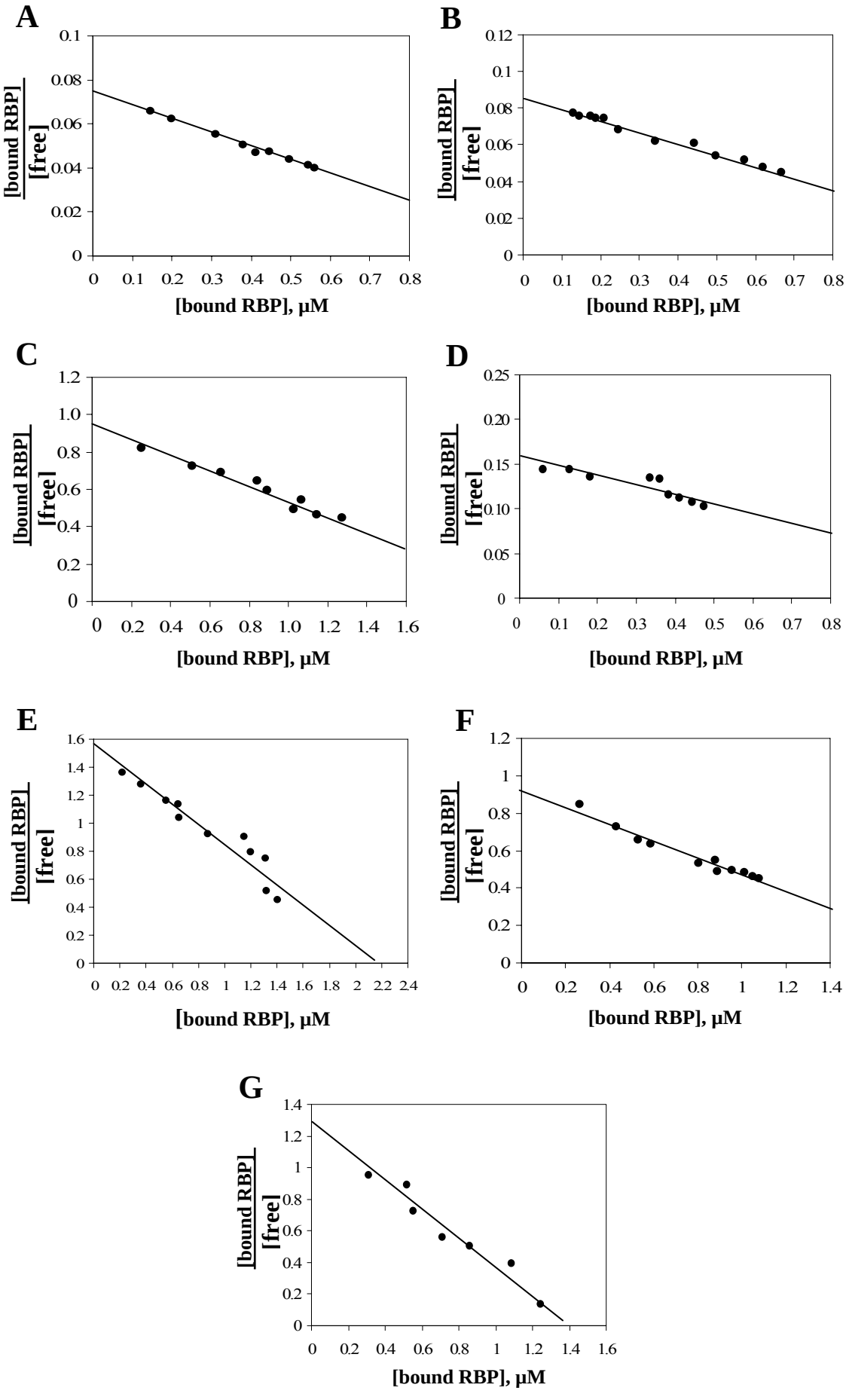

A
B
C
D
E
F
G
[bound RBP] / [free]
[bound RBP], µM

Figure 3.18 Scatchard analysis of the binding of TTRs to human RBP

TTRs and human RBP with different molar ratio were incubated at 4 °C for 1 h prior the Western blot analysis was performed. The specific binding was determined from the intensities of bound and free TTR bands. The specific binding were then plotted and dissociation constant (K_d) of the binding was calculated. At least four independent replications were carried out in each experiment. A, human TTR purified from plasma; B, croc/huTTR; C, crocTTR; D, hu/crocTTR; E, xeno/crocTTR; F, pigC/crocTTR; G, truncated crocTTR.

Table 3.4 Summarization of the K_d values for the binding of TTRs to RBP

TTR	K_d (μM)*
plasma huTTR	17.08±3.25
croc/huTTR	22.52±5.84
crocTTR	2.54±0.23
hu/crocTTR	11.31±0.07
xeno/crocTTR	1.66±0.27
pigC/crocTTR	2.34±0.45
truncated crocTTR	1.19±0.19

***** Results are presented as mean ± SD of the K_d (from 3 replicates)

9. Influence of N- and C-terminal regions on protease activity of TTRs

There are several proteases found in nature that are deeply involved in physiology and pathology. The proteolysis mechanism and the specific substrate for most these enzymes well defined. Recently, TTR was shown having a protease activity and new function that the activity may be involved has been demonstrated (Liz *et al*., 2004). However, the action of TTR as a protease has been classified into the group of cryptic enzymes, which comprised of several proteases such as secretase that involved in Alzheimer's disease (AD). The cryptic nature of TTR came from the fact that it lacks canonical structural protease determinants. In addition, its physiological function is apparently unrelated to proteolysis (for review see Liz and Sousa, 2005). There are three substrates of TTR have been revealed so far. These are apoA-I (Liz *et al*., 2008), amyloid beta peptide (Costa *et al*., 2008) and, very recently, the amidated neuropeptide Y (NPY) (Liz *et al*., 2009). Under physiological conditions, a fraction of 1-2% of TTR in human plasma co-circulates with the high density lipoproteins (HDLs) through its binding to apoA-I, a major protein component of HDL. TTR was found can cleave the C-terminus of apoA-I specifically after phenylalanine residue 225. The cleavage of apoA-I by TTR was shown not only be involved in the cholesterol metabolism but also promoted of the fibril formation of apoA-I (Liz *et al*., 2007).

TTR was suggested to be a chymotrypsin-like serine protease for apoA-I according to the finding that the general inhibitors for serine and chymotrypsin-like serine proteases inhibited the proteolytic activity of TTR. In addition, the cleavage by TTR preferred a Phe on P1 of the cleavage site and the activity was optimum at pH 6.7 (Liz *et al*., 2004). However, with the other two substrates, the exact cleavage site on the substrates is still un-identified. TTR was found act as sequester for the amyloid beta peptide (Aβ), the major component of senile plaques deposited in brain of the AD patients. The cleavage of NPY by TTR was shown related to the functions of TTR in nerve cells such as promoting the regeneration of neuritis (Liz *et al*., 2009). These evidences suggested that the important of the protease activity of TTR not only for physiological functions but also for the occurrence of some diseases.

The analysis of possible canonical catalytic triad of TTR from primary structure is difficult because TTR is a homotetrameric protein each of which monomer contains

11, 4 and 4 residues of serine, aspartic acid and histidine, respectively. Although the three dimensional analysis of the TTR subunit revealed that the arrangement of serine at position 46 was in the compatible position to a canonical catalytic triad (His Asp Ser), the site-directed mutagenesis of the serine residue was shown not involved with the protease activity of TTR. This confirmed the non-canonical serine protease nature of TTR (Dodson and Wlodawer, 1998; for review see Liz and Sousa, 2005).

RBP was shown completely inhibited the proteolytic activity of TTR (Liz *et al*., 2004). This could suggest the same or very nearby location of the binding sites of TTR for substrate and RBP. If so, the question arises whether N- and C-terminal regions of the TTR subunit have influence on the proteolytic activity of TTR. To elucidate, proteolytic activities of the recombinant TTRs and their chimeras were examined using casein and apoA-I as substrates.

The first substrate that used for quantifying the proteolytic activity of TTRs was the casein conjugated with fluorescein isothiocyanate (FITC-casein). The caseinolytic activity of the recombinant TTRs and human TTR purified from plasma were analyzed with the method of Twining (1984) with modifications as described in the Methods and Materials section 2.10. In an assay, TTR was incubated with FITC-casein for different periods. The uncleaved casein was precipitated out by TCA and the cleaved product was determined by fluorospectrophotometer. To determine the autohydrolysis of FITC-casein, the FITC-casein control, with and without precipitation by TCA, were included at each time point. The result showed that the autohydrolysis of the FITC-casein at 37^{o}C was ~30-40% over 12 h. This was not significantly impacted on the total caseinolytic activities determined from TTRs in this study.

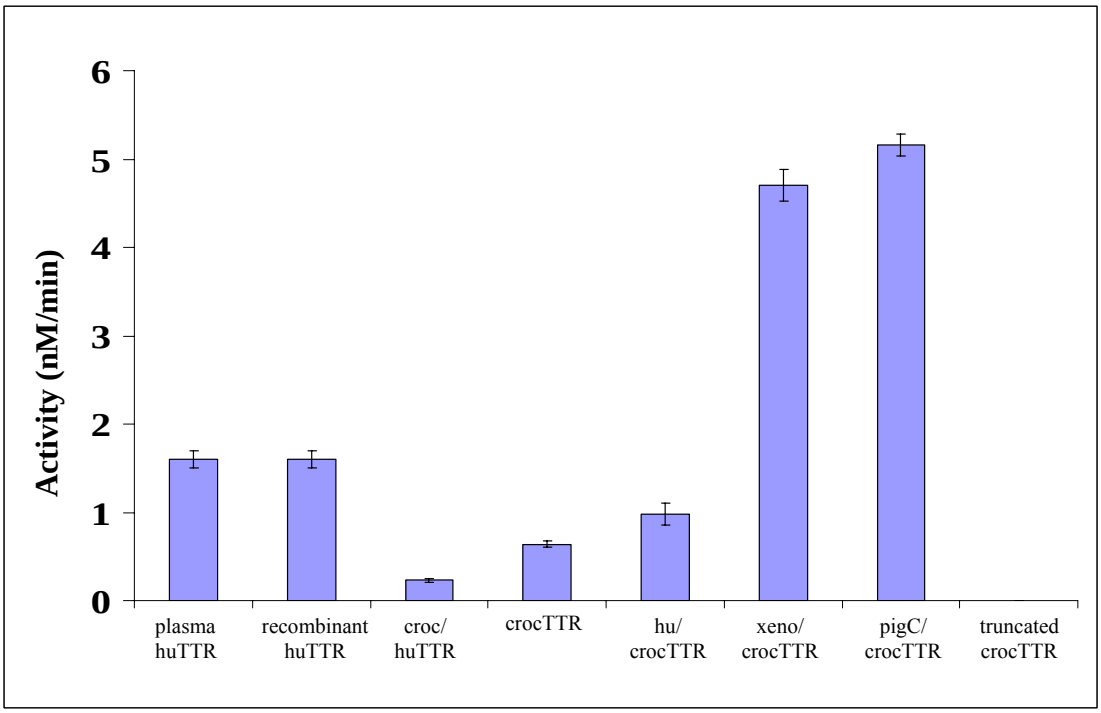
Three wild type TTRs, i.e. crocTTR, recombinant huTTR and human TTR purified from plasma, and 5 chimeric TTRs, i.e. hu/crocTTR, xeno/crocTTR, pigC/crocTTR, truncated crocTTR and croc/huTTR, were examined for their proteolytic activity. In previous study, human TTR was shown posses protease activity (Liz *et al*., 2004). The similar result obtained in this thesis. The catalytic rate of both recombinant huTTR and human TTR from plasma was 1.6±0.09 nM/min (Table 3.5), which was 2.5 times higher than that of crocTTR (0.64±0.03 nM/min) (Figure 3.19 and Table

3.5). All the recombinant chimeric TTRs except truncated crocTTR had a capability to cleave FITC-casein, though with different rates.

In comparison among wild type TTRs, the proteolytic activity of native and recombinant human TTRs was similar, but significantly higher than the wild type *C. porosus* TTR. The chimeric huTTR, i.e. croc/huTTR, had a significant lower activity than the wild type. The chimeric crocTTRs, on the other hand, showed differences in extent of the activity in comparing to the wild type crocTTR. While xeno/crocTTR and pigC/crocTTR had very high activity, hu/crocTTR showed similar activity to the wild type and truncated crocTTR did not have activity (Figure 3.19A) though higher amount of the TTR was attempted (data not shown).

It seemed like that when the N-terminal region of human TTR was changed to that of crocTTR (that found in croc/huTTR), the TTR lost its proteolytic activity and this loss was ~86% in comparing to the wild type TTR (Figure 3.19B). The crocTTR that the N-terminal region was changed to that of human TTR (hu/crocTTR) or lacked N-terminus (truncated crocTTR) did not have or changed in ability to digest the FITC-casein. However, the proteolytic activity extraordinary increased when the N-terminal sequence was changed to that of *X. laevis* TTR (xeno/crocTTR) or the C-terminal region was altered. The activity of hu/crocTTR increased to only 1.53 folds (V_0 was 0.98±0.12 nM/min) of the wild type crocTTR, while the activities of xeno/crocTTR and pigC/crocTTR were 7.34 folds (V_0 was 4.7±0.18 nM/min) and 8.06 folds (V_0 was 5.16±0.12 nM/min) of the wild type crocTTR, respectively (Figure 3.19B, Table 3.5).

A


Activity (nM/min)
0
1
2
3
4
5
6
plasma huTTR
recombinant huTTR
croc/ huTTR
crocTTR
hu/ crocTTR
xeno/ crocTTR
pigC/ crocTTR
truncated crocTTR

B

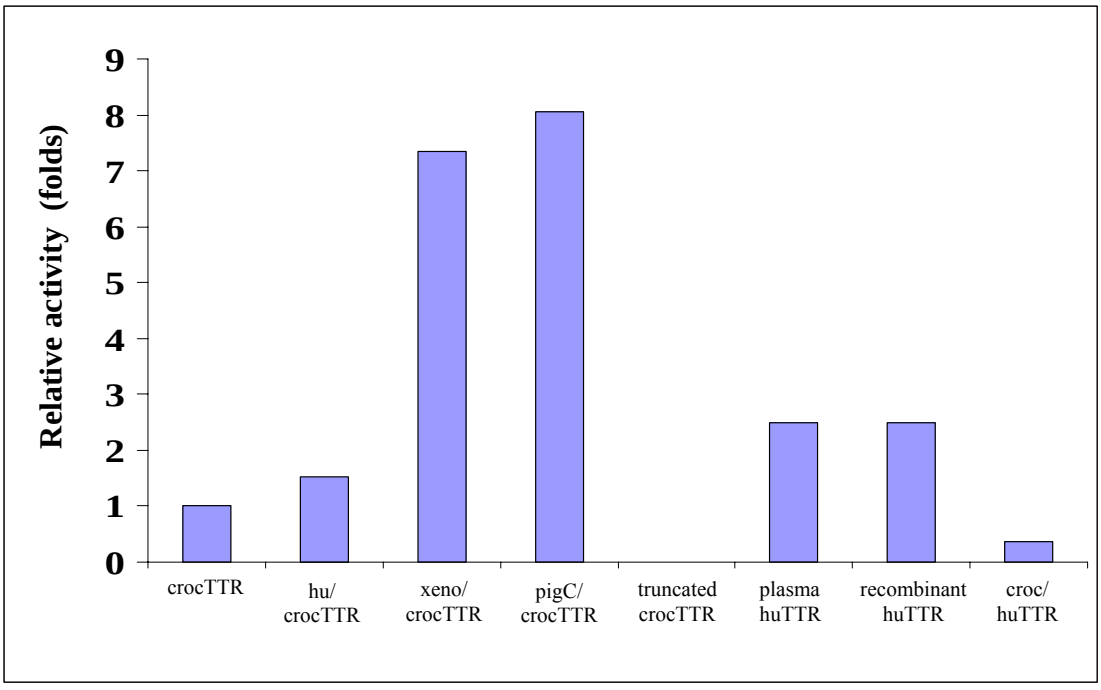

Relative activity (folds)
0
1
2
3
4
5
6
7
8
9
crocTTR
hu/ crocTTR
xeno/ crocTTR
pigC/ crocTTR
truncated crocTTR
plasma huTTR
recombinant huTTR
croc/ huTTR

Figure 3.19 The proteolytic degradation of FITC-casein by TTRs

The recombinant TTRs or TTR from human plasma (plasma huTTR) were incubated with FITC-casein at 37°C for different periods. Uncleaved FITC-casein was then precipitated with 5% TCA, and fluorescence intensity of cleaved product in the clear solution was measured at exitation wavelength of 490 nm and emission wavelength of 525 nm. One relative fluorescence unit equals the fluorescence of 0.006 μM quinine hydrochloride. (A), the proteolytic activity of each TTR; (B), the relative activity of each TTR to that of crocTTR.

Table 3.5 Summarization of the proteolytic activity of TTRs using FITC-casein as substrate

TTR	Activity(nM/min)*	Relative activity to crocTTR (folds)
plasma huTTR	1.6±0.09	2.5
recombinant huTTR	1.6±0.09	2.5
croc/huTTRTTR	0.23±0.02	0.36
crocTTR	0.64±0.03	1
hu/crocTTR	0.98±0.12	1.53
xeno/crocTTR	4.70±0.18	7.34
pigC/crocTTR	5.16±0.12	8.06
truncated crocTTR	0	0

* Results are presented as mean ± SD of the enzyme activtity (from 3 replicates)

The proteolytic activity of the wild type and chimeric TTRs could be identified using FITC-casein, a universal substrate for proteases. By comparing the activity, the influence of N- or C-terminal region could be demonstrated. However, the question still arises whether the similar influence would be with *in vivo* substrates such as apoA-I. Both human TTR purified from serum and the recombinant human TTR produced using the heterologous protein expression system of *E. coli* were demonstrated have a cleavage ability to apoA-I (Liz *et al*., 2004). In this thesis, the ability to cleave apoA-I has been first attempted with human TTR purified from plasma.

The TTR was allowed to digest apoA-I at 37 °C and then cleaved and uncleaved apoA-I were analyzed by SDS-PAGE on 20% polyacrylamide gel. It revealed that apoA-I was cleaved after incubation for 1 h (Figure 3.20). Two fragments corresponding to uncleaved and cleaved (with faster mobility) apoAI were observed after incubation in the presence of TTR and clearly separated from each other on the 20% polyacrylamide gel. After incubation for longer than 3 h, intensity of the cleaved fragment became very low and other smaller fragments were observed (data not shown). In contrast, there was no cleavage or degradation of apoA-I could be observed in the reaction without TTR even incubation was carried on for longer than 3 h. This should imply step by step of the cleavage, i.e. the apoA-I was specifically cut into fragment, later on the cleaved fragment was more susceptible to the degradation by the TTR but with a non-specific manner. In order to confirm that the cleaved fragment came from apoA-I, the Western analysis using antiserum raised against human apoA-I was performed. In the control reactions, without TTR or with TTR but incubated for 0 h, only a single protein band specifically bound to the apoA-I antibody were detected. In the presence of TTR, two protein bands were observed, one with the same mobility to that observed in the control reactions and the other moved faster (Figure 3.20). Therefore, based on the specific interaction of the antibody, the smaller fragment could be identified as a part of apoA-I and corresponding to the cleaved fragment. Since the cleaved fragment non-specifically degraded by time, changes in amount of the uncleaved rather than cleaved fragments was used in elucidating the activity of TTR.

The apoA-I cleavage activity of other TTRs were then assesed with the same strategy as mentioned above in oder to elucidate the influence of N- and C-terminal regions of TTR. Each TTR was incubated with apoA-I for different periods. Then the cleavage ability was determined from intensities of the uncleaved fragment that was still remained at each incubation period. Figure 3.21 showed the kinetic cleavages of apoA-I by TTRs. The similar pattern of the apoA-I fragment was observed in all reactions with TTR even from different sources. The amount of uncleaved apoA-I decreased during incubation, however, with different rates. Two chimeric crocTTR i.e. xeno/crocTTR and pigC/crocTTR showed the fastest rate of the cleavage, and this was remarkably different from the other TTRs. The cleavage by these two TTRs almost complete after the reactions were carried on for 30 to 60 min (Figure 3.21), while the other TTRs took longer than 180 min. In addition, degradation of the cleaved fragment could be detected in very short after the cleaved fragment appeared, suggesting to an obvious high protease activity of the proteins. The cleavage rates of another two chimeric TTRs, i.e. croc/huTTR and truncated crocTTR, were slowest in comparing to all the examined TTRs. The cleaved fragment was first observed after incubation for 180 to 360 min (Figure 3.21). The intensity of the uncleaved fragment was still intense even incubation had been carried on longer than 360 min.

To evaluate for the activity, so the abities in cleavage of all examined TTRs could be compared, intensity of the uncleaved fragment was measured and amount of protein was calculated and plotted against the incubation time. The clavage rate could be directly obtained from slope of the curve. Since the protein band was detected by Coomassie blue staining, intensity of the protein band in different gel could be varied depending on several factors including amount of dye, compositions of gel and the intensity measurement. To correct and make all experiments could be compared, apoA-I only was incorporated into each experiment and intensity of the uncleaved fragment was extrapolated according to the apoA-I control. Figure 3.22A demonstrated the kinetic decrease of the uncleaved apoAI in the presence of crocTTR and its chimeras. These results similar indicated to those directly observed from gel. The xeno/crocTTR and pigC/crocTTR had the highest rate for cleaving apoAI in comparing to the others. Amount of the uncleaved apoA-I dramatically decreased during incubation for the first 30 min. Less than 50% and 6% of the uncleaved apoA-I

remained in the reaction with xeno/crocTTR and pigC/crocTTR, respectively. In contrast, only small amount of the uncleaved apoA-I decreased after incubation for 30 min in the presence of trancated crocTTR (Figure 3.22A), and the cleavage appeared with slow rate. Almost 70% of the uncleaved apoA-I still remained after the reaction occurred for 360 min. The cleavage ability of hu/crocTTR was similar to that of crocTTR, and the clevage rate was in between pigC/crocTTR and truncated crocTTR. In comparing to human TTR purified from plasma, the recombinant huTTR had a slightly higher cleavage ability to apoA-I. However, 1.5 to 2 folds lower in the cleavage was observed in the reaction with croc/huTTR (Figure 3.22B). This should imply negative influence of the N-terminal region on the apoA-I cleavage of TTR.

The initial velocity (V_0) of each TTR was calculated and shown in Figure 3.23A. These values confirmed the results obtained from the direct observation from gel and the kinetic decrease of the substrate. The V_0 for apoA-I of the recombinant huTTR was 17.07±0.64 nM/min, while that of the plasma human TTR was 8.99±0.77 nM/min, confirming slightly higher activity of the recombinant TTR. However, the V_0 of croc/huTTR was 5.79±0.50 nM/min, which was 1.5 to 3 folds lower in comparing to plasma human TTR and recombinant huTTR, respectively. The crocTTR had V_0 for apoA-I of 12.77±2.05 nM/min. This was not much different from those of human TTR, either native or recombinant. In comparison, the V_0 of hu/crocTTR (9.91± 1.11 nmole/min) was similar to that of the wild type TTR. The extraordinary high V_0 was observed in xeno/crocTTR and pigC/crocTTR. The V_0 of these two chimeric TTRs were 30.48±3.94 nM/min and 46.73±1.75 nM/min, respectively. These were 2.39 (xeno/crocTTR) and 3.66 folds (pigC/crocTTR) greater than the wild type crocTTR (figure 3.23B). The truncated crocTTR, on the other hand, had very low V_0 for apoA-I. It was 3.86±1.62 nM/min, which was 0.30 folds lower than that detected in crocTTR. The V_0 of each TTRs were also summarized in table 3.6.

The determination of the proteolytic activity of TTR to FITC-casein and apoA-I revealed the similar influence of N- and C-terminal regions on both activity. The alteration in the proteolytic activity detected in croc/huTTR clearly demonstrated that changing the N-terminal sequence of human TTR from shorter and more hydrophilic to longer and more hydrophobic could lead to reduction of the activity. Whereas, the

experiment with xeno/crocTTR and pigC/crocTTR revealed that either changing the N- or C-terminal sequence of crocTTR to longer and more hydrophobic could lead to increase the proteolytic activity of the TTR.

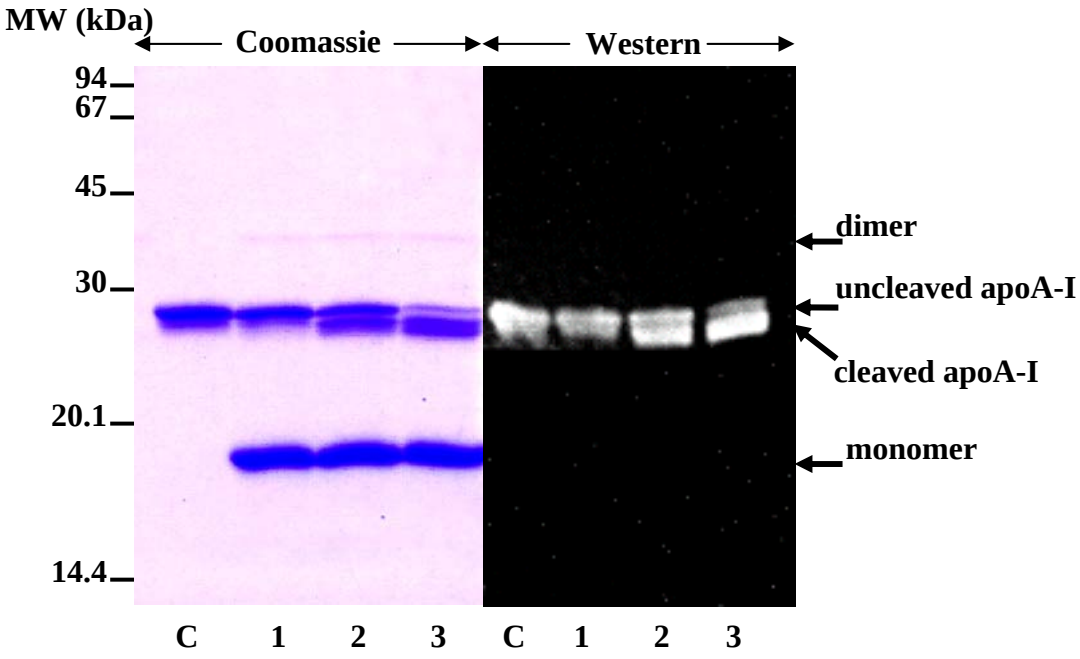


Figure 3.20 Western blot analysis of the ApoA-I cleavage by human TTR from plasma

ApoA-I (1 μg) and human TTR (2 μg) were incubated at 37^{o}C , then the reactions were resolved by SDS-PAGE (20% resolving gel) prior the protein bands were transferred to nitrocellulose membrane. The proteins were detected by staining with Coomassie blue (Coomassie) or identified with antiserum (Western). The membrane was incubated with rabbit antiserum raised against ApoA-I (1:1000) and, then, anti-rabbit IgG conjugated with horseradish peroxidase (1:10,000). Detection was performed using ECL. C, the control reaction contained ApoA-I alone; 1 to 3, the reaction mixture of TTR and apoA-I incubated for 0, 60 and 180 min, respectively. Positions of TTR monomer (monomer) and dimer (dimer), cleaved and uncleaved apoA-I were indicated. Molecular weight of the protein markers also shown.

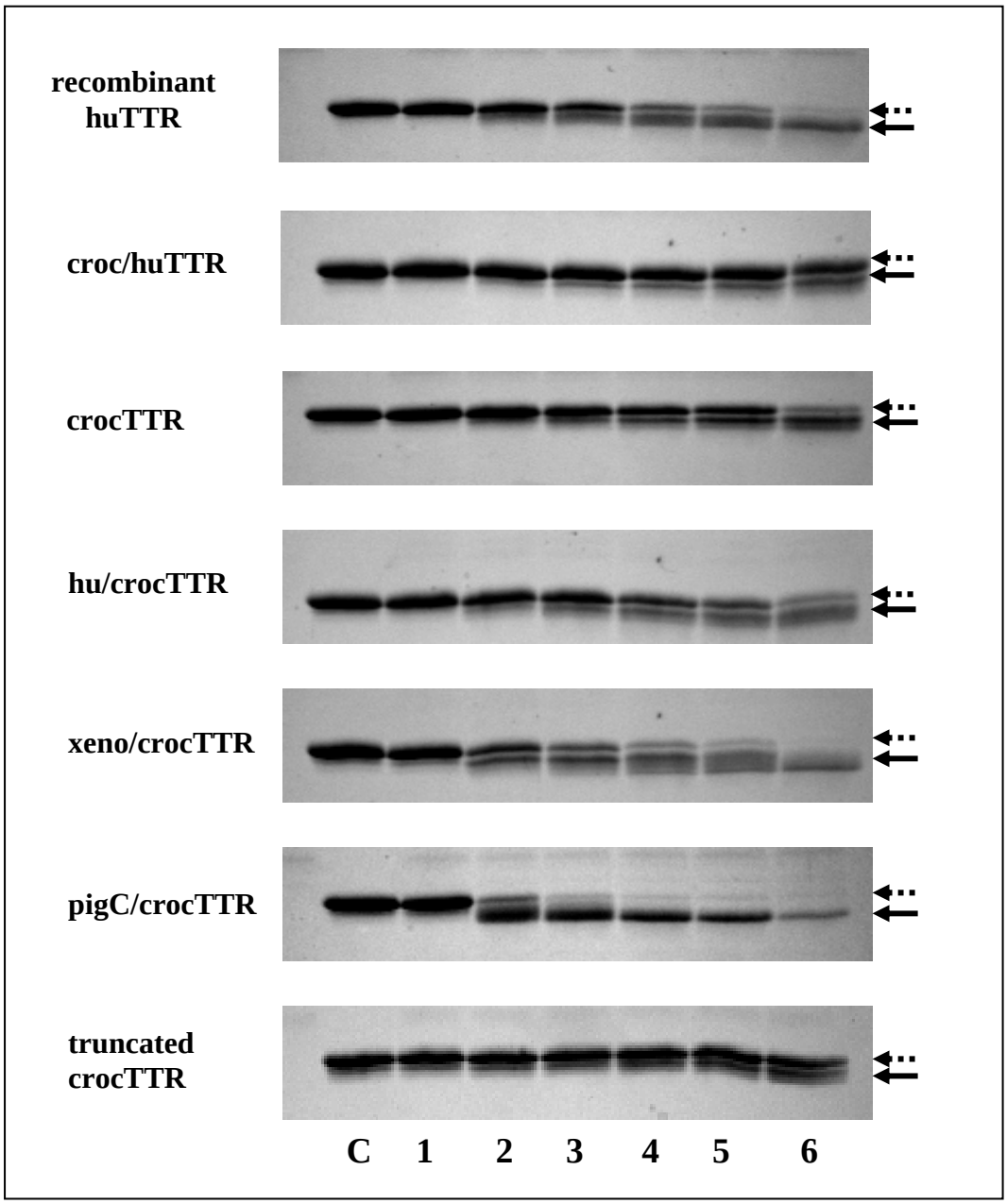
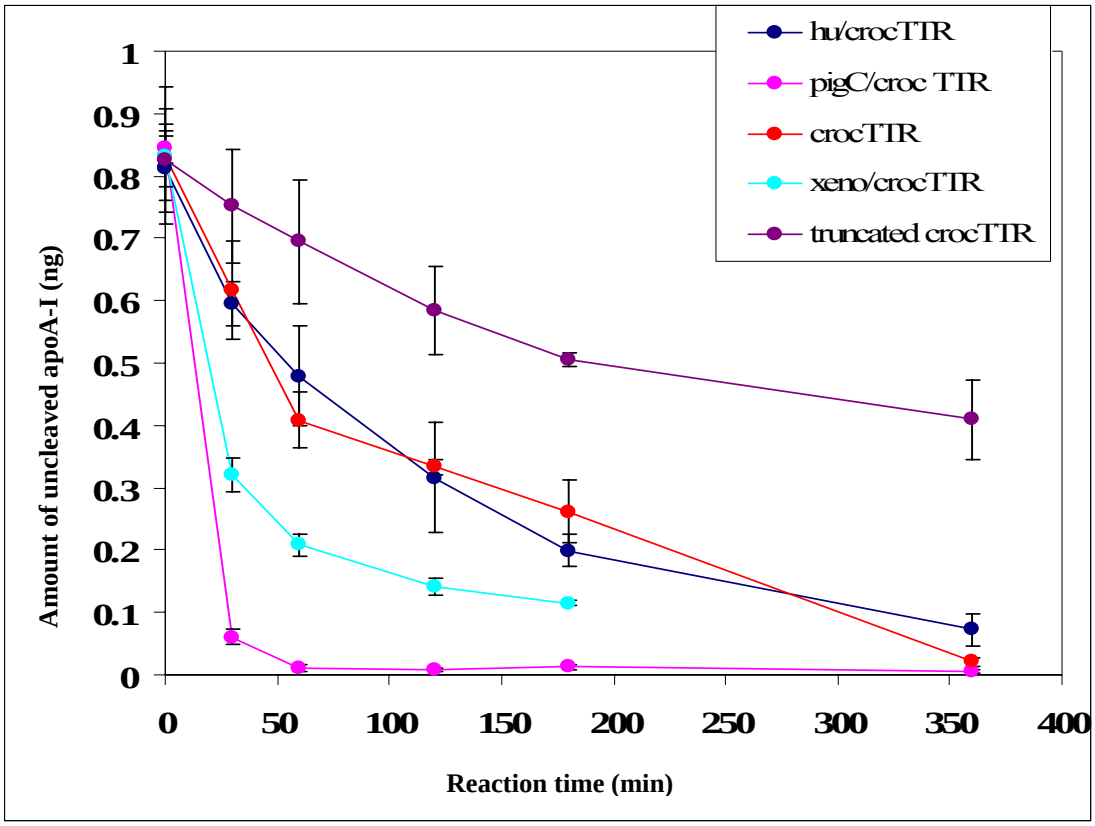


Figure 3.21 Analysis of apoA-I cleavage by TTRs

Aliquots of ApoA-I (1 μg) and TTR (2 μg) were incubated at 37°C for 6 h. Then reaction was terminated and analyzed by SDS-PAGE on 20% polyacrylamide gel. The proteins were then detected by staining with coomassie blue. C, the control in which contained apoA-I alone; 1 to 6, reaction that the incubation was terminated at 0, 30, 60, 120, 180 and 360 min, respectively. Uncleaved and cleaved apoA-I were indicated by dot and solid arrows, respectively.

A


hu/crocTIR
pigC/croc TIR
crocTIR
xeno/crocTIR
truncated crocTIR
Amount of uncleaved apoA-I (ng)
Reaction time (min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
50
100
150
200
250
300
350
400

B

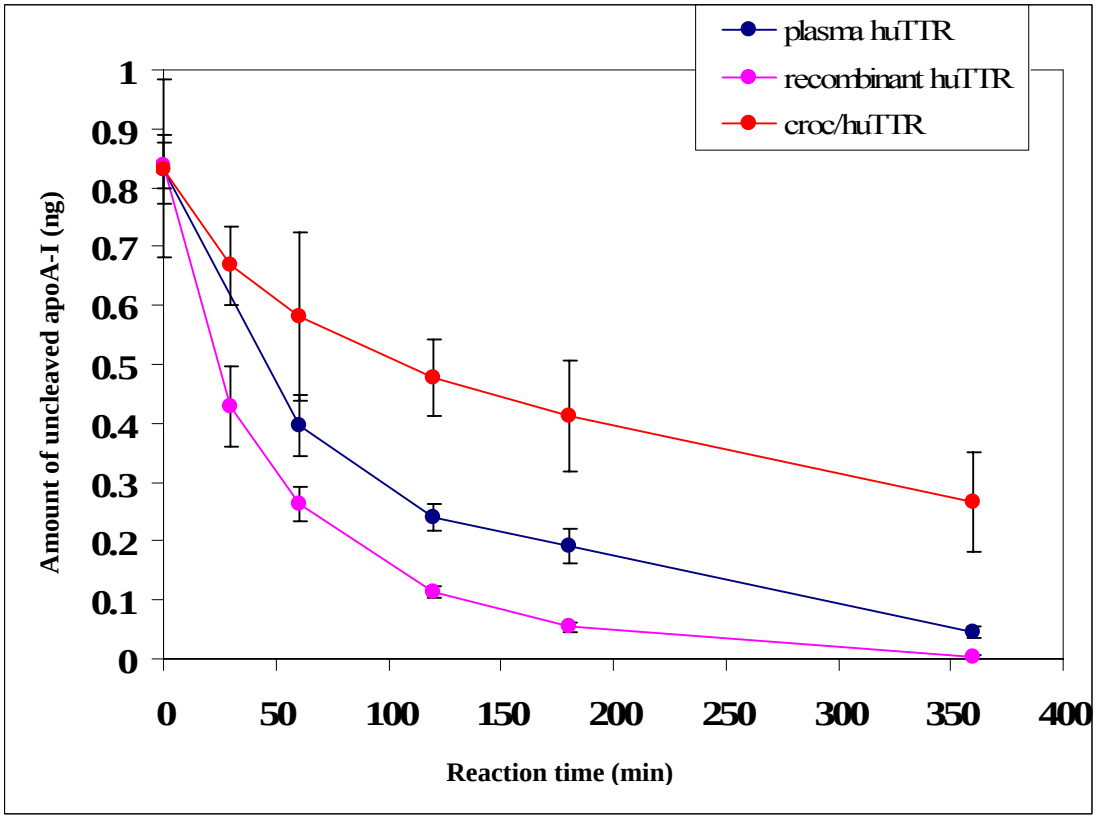
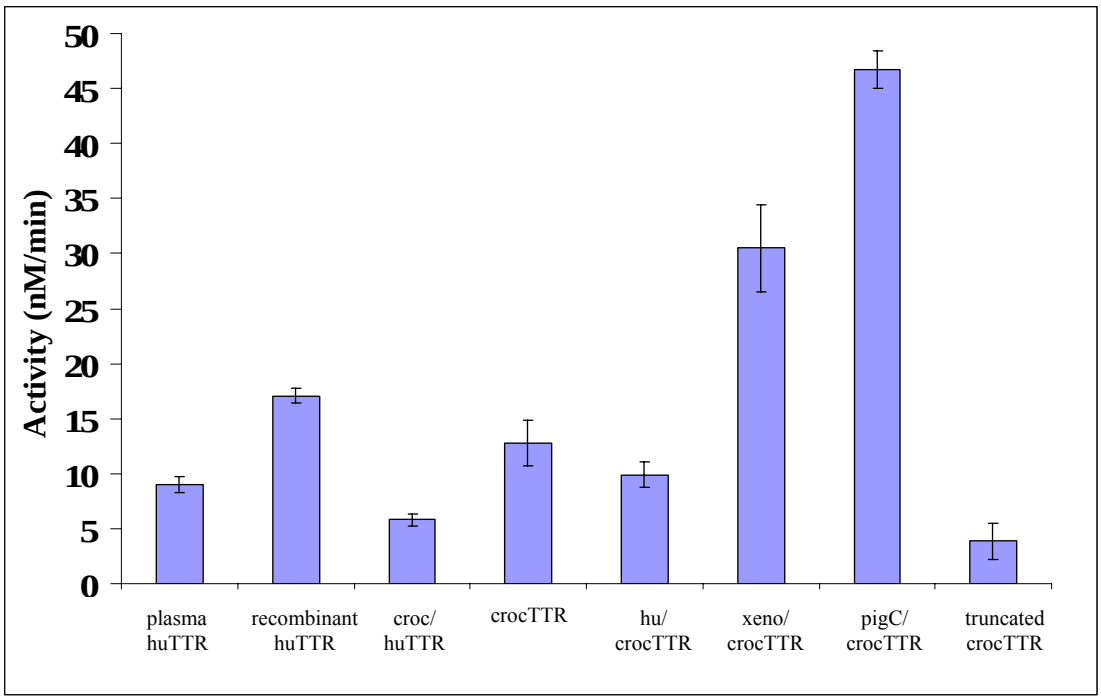

plasma huTIR
recombinant huTIR
croc/huTIR
Amount of uncleaved apoA-I (ng)
Reaction time (min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
50
100
150
200
250
300
350
400

Figure 3.22 The kinetic cleavage of apoA-I by TTRs

The recombinant TTR was incubated with apoA-I at 37^{o}C and the reaction was terminated at six time points (0, 30, 60, 120, 180 and 360 min). Then, the intensities of uncleaved apoA-I were measured and amount of protein were calculated and plotted. The kinetic cleavages of wild type crocTTR and its chimeras (A) and those of wild type huTTR and its chimeras (B) were shown.

A


Activity (nM/min)
50
45
40
35
30
25
20
15
10
5
0
plasma huTTR
recombinant huTTR
croc/ huTTR
crocTTR
hu/ crocTTR
xeno/ crocTTR
pigC/ crocTTR
truncated crocTTR

B

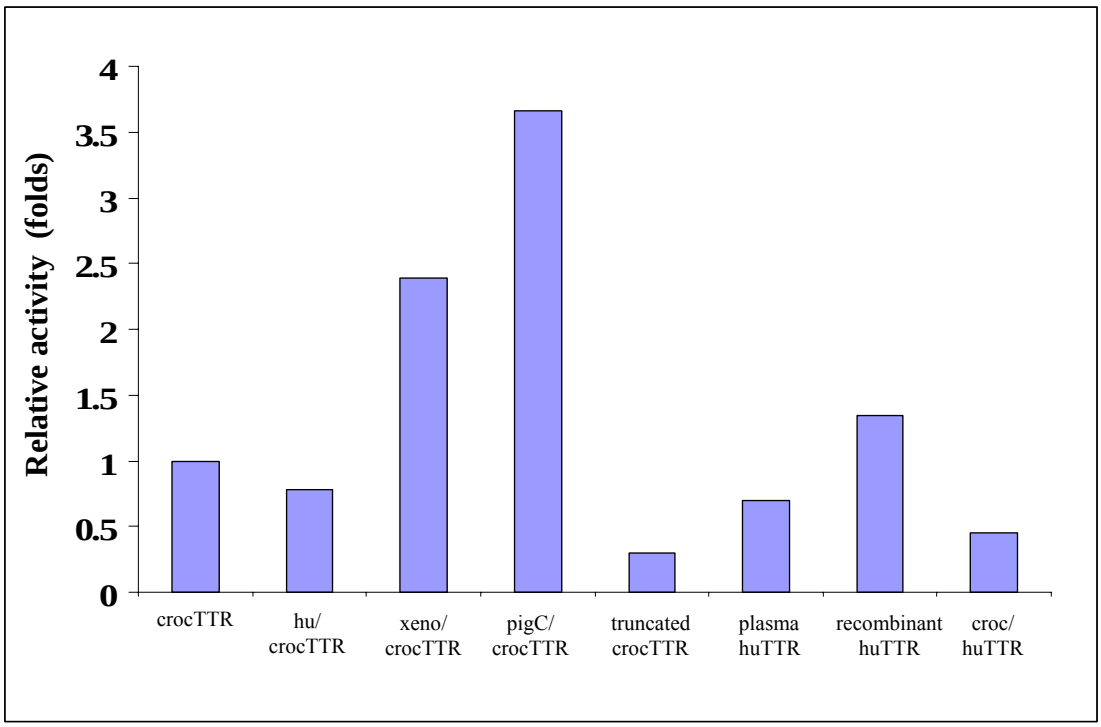

Relative activity (folds)
4
3.5
3
2.5
2
1.5
1
0.5
0
crocTTR
hu/ crocTTR
xeno/ crocTTR
pigC/ crocTTR
truncated crocTTR
plasma huTTR
recombinant huTTR
croc/ huTTR

Figure 3.23 The influence of N- and C-terminal regions of TTR on the cleavage of ApoA-I

TTRs were incubated with apoA-I at 37^{o}C for different periods. Then, the cleavage of ApoA-I was analyzed. (A), the proteolytic activity of each TTR; (B), the relative activity of each TTR to that of crocTTR.

Table 3.6 Summarization of the ApoA-I cleavage activity of TTRs

TTR	Activity (nM /min) *	Relative activity to crocTTR (folds)
plasma huTTR	8.99±0.77	0.70
recombinant huTTR	17.07±0.64	1.34
croc/huTTR	5.79±0.50	0.45
crocTTR	12.77±2.05	1
hu/crocTTR	9.91±1.11	0.78
xeno/crocTTR	30.48±3.94	2.39
pigC/crocTTR	46.72±1.75	3.66
truncated crocTTR	3.86±1.62	0.30

* Results are presented as mean ± SD of the enzyme activtity (from 3 replicates)

CHAPTER 4

DISCUSSION

1. Expression of the recombinant TTRs in yeast *P. pastoris*

Study of the relationship between structure and functions of TTR is a purpose of this thesis. In order to reach this goal, synthesis of the recombinant TTRs with differences in amino acid sequence was required with several reasons. First, the expression of TTR gene in some vertebrate species occurs only in a specific stage of life such as in crocodile, the TTR gene is expressed in liver of the animal only in early stage of life and the expression tissue will move from liver to choroid plexus in the animal adult (Prapunpoj *et al*., 2002). It seems like one can isolate TTR from any stage of the animal, though from different tissues. However, TTR expressed only small amount in choroids plexus, makes it is difficult to obtain sufficient amount of the native TTR for study. To overcome this limitation, the heterologous protein expression system is required. Second, it is well known that there are other THDPs i.e. albumin and TBG in plasma of vertebrates and these proteins form a network system. The strong redundancies in the network that is involved in determining TH distribution, which TTR is part, renders the system so effective. The deficiency in one component can be compensated *in vivo* by changes in the raise of the other THDPs. This character makes it difficult to quantitatively analyze the relationship between TTR structure and function *in vivo* using genetic alterations or specific inhibitors. Third, it is not possible to reveal influence of the N- or C-terminal region on functions of TTR via study of the three dimensional structure of the TTR subunit since the first 10 amino acids of the TTR subunit do not form an order structure and locates outside the tetrameric structure of TTR (Blake *et al*., 1978 and Sunde *et al*., 1996). Construction and synthesis of the recombinant chimeric TTRs containing variations in the N- or C-terminal structure is the most appropriate. The functional properties of such chimeric TTRs could then be analyzed and insight of the relationship between structure and function could be revealed.

The heterologous protein expression system of *P. pastoris* was used for synthesis of the recombinant TTRs used in this thesis. These TTRs included hu TTR,

croc/huTTR, hu/crocTTR, pigC/crocTTR and truncated crocTTR. The *Pichia* system was chosen to produce TTRs because of many advantages that yeast has over other eukaryotic hosts (Cregg *et al*., 1993). Yeast grow fast even in a simple medium similar to other prokaryotes such as *E. coli*. As being an eukaryote, yeast has similar post-translational modifications as those exist in vertebrates. The heterologous protein synthesized by yeast is secreted to the medium outside the yeast cells facilitating in purification of the protein.

In wild type *P. pastoris* strain GS115 and SMD1168, the expression of TTR gene and the secretion of TTR protein are driven by a strong promoter, *AOX1*. The expression "cassette" consisting of signal sequence plus TTR gene was inserted into the yeast genome at *HIS4* locus. The integration was success by linearization the expression vector pPIC3.5 and pPIC9 with *Sal*I prior the transformation was carried on. The transformation efficiency obtained in this thesis was similar to that previously reported (Scorer *et al*., 1994) revealed to efficiency of the competent cells used. The His^+Mut^+ transformants were the majority clones obtained. The synthesized TTR was found secreted into the culture medium suggesting an effective function of the signal sequence, either of the TTR itself presegment or that of α-factor. In this thesis, the synthesis of the recombinant human TTR was attempted using two *Pichia* expression vectors, pPIC3.5 and pPIC9. Whereas, only pPIC9 was used to express the chimeic TTRs. The recombinant TTR synthesized in *Pichia* was extracellularly secreted out of the yeast cells via native signal sequence of TTR itself if the gene was ligated into pPIC3.5, and via signal sequence of yeast α-factor protein if the gene was ligated into pPIC9. The recombinant huTTR was only successfully synthesized by using its signal sequence in pPIC3.5 suggested that the signal sequence in the constructed vectors have different efficiency for the insertion of the protein into the endoplasmic reticulum of yeast. However, the other four chimeric TTRs, which three of them were originated from *C. porosus* TTR and one of them was originated from human TTR, were synthesized and secreted efficiently by using pPIC9. The reason of the difference in protein secretion and production using pPIC9 between human TTR and chimeric TTRs is still unknown. The compatibility in sequence between the DNA insert and the vector is one of a possible reason. This will be more discussed in next paragraph.

The amount of recombinant TTRs secreted by yeast was highly dependent on the composition of growth medium. It has been demonstrated that the amount of secreted protein will increase when the buffered medium was used (Clared *et al*., 1991). In addition, the presence of substrates such as the peptide components of yeast extract and peptone reduced the action of pH-dependent extracellular protease in *P. pastoris* and consequently decreases the proteolytic degradation of secreted recombinant proteins (Clare *et al*., 1991). Therefore, in this thesis, the buffered medium including BMGY and BMMY were used for growth and induction for the recombinant TTR synthesis by yeast. The kinetic secretion in large scale among the recombinant TTRs were different. In comparison, the *Pichia* clones, except that for truncated crocTTR, those contained the nucleotide sequence encoding for a part of amino acid sequence of *C. porosus* TTR produced TTRs in large amount. Approximate 60 to 200 mg of the TTRs was synthesized and secreted out to 1 liter of culture medium. There are two possible reasons. First, the nucleotide sequence encoding for *C. porosus* TTR is probably highly compatible to the promoter and other nucleotide sequences required for the functioning and maintaining of the expression vector in yeast cells. Second, the nucleotide sequence of *C. porosus* TTR may facilitate integration as well as increase probability of multiple insertion of the vector into yeast chromosome. The multiple gene insertion at a single locus in *Pichia* could occur spontaneously, however with low efficiency for 1%-10% of His^+ transformants (Clare *et al*., 1991). It is convincible that the recombinant *Pichia* clones for crocTTR and its chimeras except truncated crocTTR received multiple copies of the TTR genes, therefore, these clones produced and secreted in large amount of the recombinant proteins than that obtained only single copy gene. The recombinant clone for truncated crocTTR, on the other hand, produced in a low amount of the recombinant protein though the clone contained the expression vector ligated to a gene fragment of crocTTR. This might be due to this TTR lacked of the N-terminal region, thus, had a high tendency to form aggregates rather than homotetramer in the yeast cells leading to only small amount of the protein secreted into the culture medium.

One of the advantages in using the expression system of *Pichia* is only small amount of yeast endogenous proteins was secreted into culture medium. The heterologous protein is secreted as a major protein in medium (For examples see

Tschopp *et al*., 1987 and Barr *et al*., 1992). This facilitates purification of the foreign protein from other proteins. In this thesis, the recombinant TTRs could be able to isolate from other proteins in culture medium with a single step by preparative native-PAGE (e.g. crocTTR and hu/crocTTR, which secreted in large amount) or with two steps by preparative native-PAGE and followed by affinity chromatography on human RBP-Sepharose (e.g. huTTR and truncated crocTTR, which secreted in only small amount). In comparing to a single step by affinity chromatography on human RBP-Sepharose as previous reported (Prapunpoj *et al*., 2002), the purification strategies used in this thesis provided less amount of the purified recombinant TTRs. Approximate 1 to 10 mg of TTR could be purified from 1 liter of yeast culture. Loss of the protein during purification could occur in several steps including concentration of the protein by ultrafiltration. However, only a single or two steps of purification was required to obtain the purified TTRs produced by *P. pastoris*, which was more benefit than using the heterologous protein synthesis system of *E. coli*. Since the recombinant protein was intracellular synthesized and often stored in a clusion body, several steps of purification are needed (Furuya *et al*., 1989; Murrel *et al*., 1992; Berni *et al*., 1994 and Rosen *et al*., 1994). Cell disruption and extraction with some strong chemicals such as detergents lead to denaturation of protein. Although the chemicals may be removed out and renaturation of the protein occur, most of proteins in particular those contain multiple subunits have incomplete folding and, thus, improper functioning.

In order to reveal the quality of the recombinant TTR produced by *P. pastoris*, the physicochemical properties of the native and the recombinant TTRs were examined. The electrophoretic mobility on a non-denaturing polyacrylamide gel at pH 8.6 of all recombinant TTRs was faster than that of albumin. This agrees with the typical characteristic of most vertebrate TTRs found in nature (Richardson *et al*., 1994). Slightly difference in the mobility was observed in pigC/crocTTR and truncated crocTTR. These two TTRs, though moved faster than albumin, migrated slower than other TTRs in nature. Difference in total charges of these two chimeric TTRs might be a reason for the slow mobility. Masses of the recombinant TTR subunits were determined by SDS-PAGE. The results showed slightly different masses between the TTRs originated from human TTR and those originated from crocTTR.. The subunit

masses of the recombinant human TTR and its chimera, i.e. croc/huTTR, was ~17 kDa (Table 3.2) similarly to that of the human TTR purified from plasma. However, these masses were slightly different from that direct obtained from the deduced amino acid sequence (~14 kDa) (Table 3.2). The recombinant crocTTR and its chimeras except truncated crocTTR had the subunit masses ~15 kDa (Table 3.2). Different from other recombinant TTRs, truncated crocTTR sometimes showed two addition discrete bands on SDS-polyacrylamide gel (Figure 3.12) demonstrated incorrect recognition and cleavage of the *S. cerevisiae*-derived α-factor prepro segment possible occurred, leading to shorter or longer segment of the N-terminus. However, by amino acid sequencing of the truncated crocTTR N-terminal region, no addition of amino acid sequence was detected.

Tetrameric structure of TTR is important for its proper function in particular binding to THs. To reveal whether or not TTR subunit had a correct folding and forming to tetramer, molecular weight of the TTR molecule is needed. The recombinant TTRs synthesized by *Pichia* were determined for the molecular mass by HPLC using gel–permeation column. It showed that all the recombinant TTRs had the similar molecular masses to those reported for native TTRs from many vertebrate species (Yamauchi *et al*., 1993; 1999 and Chang *et al*., 1999). Although truncated crocTTR showed three discrete bands on SDS-polyacrylamide gel sometimes, only a single peak with a molecular weight corresponding to TTR tetramer was observed by HPLC. In addition, the immunochemical reaction with antibody raised against TTR confirmed that these two addition discrete protein bands were TTR monomer. Eukaryotic posttranslational events, such as signal sequence processing, protein folding, disulfide bridge formation, and glycosylation, take place during proteins passing through the secretory pathway. Posttranslational modifications often involve the addition of oligosaccharides to O- and N-asparagine-linked sites on proteins. The types of sugar in the oligosaccharides, size and structure of these polymers, can all affect physicochemical properties and the ability of a protein to fold, and consequently its activity, and immunogenicity. Little information is available on glycosylation in *P. pastoris*. Addition of carbohydrate moieties to foreign proteins can occur though the protein is not glycosylated at all in its native host (Brierley, 1994). The amino acid sequence of TTR contains at least one site for a possible

glycosylation. However, no glycosylation is detected in native TTRs. Therefore, post-translational modification in particular glycosylation might be an explanation for appearing of the discrete bands. The mass of the recombinant TTR determined by gel filtration chromatography was four times to mass of the subunit determined by SDS-PAGE, indicating that the recombinant TTRs existed in the form of a tetramer similar to native TTR.

Native TTR in plasma exists in the tetrameric form. Part of it can remain as a dimer after denaturation by SDS (Dickson *et al.,* 1982 and Furuya *et al.,* 1989). This feature was also found in the recombinant TTRs produced by *Pichia* studied in this thesis. Recombinant TTR migrated as a band in the position corresponding to the dimer in SDS-PAGE after boiling for 20 min in the presence of 2 % SDS and 2.5 % β-mercaptoethanol, demonstrating the strength of dimer-dimer interaction in TTR. This dimer was confirmed to be TTR by the interaction with the specific antibodies against TTR (Figure 3.14).

The N-terminal amino acid sequence of the subunits of secreted TTRs purified from *Pichia* cultures were determined to identify whether they were identical to the TTR as predicted from their nucleotide sequences. The recombinant human TTR and truncated crocTTR showed no extra amino acid at the N-termini, demonstrated clearly that the signal sequence of TTR itself, in human TTR, and the yeast-derived α-factor prepro segment, in truncated crocTTR, (Julius *et al.,* 1984 and Clare *et al.,* 1991) were correctly recognized and the leader sequence was accurately and efficiently cleaved in *P. pastoris*. Although two addition amino acid residues were detected at the N-terminal sequence of hu/crocTTR, this contrary to the recombinant TTRs obtained from the *E. coli* system. The presence of seven extra amino acid residues (Furuya *et al.,* 1989) or methionine providing the initiation site for translation in prokaryotes (Murrell *et al.,* 1992) was reported for TTRs synthesized in the *E. coli* system. Though these features did not affect the folding of the molecule since the N-terminus of TTR is located at the outside of the molecule, it could influence the function of the N-terminal region of TTR.

2. Influence of the N-terminal region on the binding to THs of TTR

Up to date, the specific function of TTR particular in brain is still discussed. However, the most well known physiological functions of TTR is the transporter for THs and retinol, in the latter through binding to retinol binding protein or RBP. Binding to THs is one of the most interesting functions of TTR. The binding of TTR to THs was first evidenced by Ingbar (1958) in human serum. Thereafter, TTR from several vertebrate species including mammalians, birds, reptilians, amphibians and fish (Larsson *et al*., 1985; Richardson *et al*., 1997; for review see Schreiber and Richardson, 1997 and Santos and Power, 1999) were studied for its ability to bind THs. The binding affinity for THs of TTRs varied among vertebrate species as shown in Table 4.1. Eutherian and Marsupial TTRs bound T_4 with higher affinity than T_3, however with less strength in Marsupial TTRs. The avian TTRs bound T_3 with higher affinity than T_4, similarly to reptilian, amphibian and fish TTRs. It seems likely that during evolution, TTR lost its affinity to bind T_3 in lower vertebrates but change to be a carrier of T_4 in mammals (Power *et al*., 2000 and Morgado *et al*., 2008). This evolutionary change is consistence with the adaptation regarding THs transport in which T_4 act as a prohormone that secreted from thyroid gland and then is converted to T_3 in specific tissue by specific deiodinases of higher vertebrates. As already mentioned in review section that changes in binding affinity to THs during evolution was related to the changes in length and hydropathy of the N-terminus of TTR molecule. The N-terminal region is defined as the amino acids from the N-terminus until the Cys residue which is the first to be unambiguously defined by electron density in X-ray crystal structures (i.e. Cys10 in human TTR) and considered part of the core structure of TTR. Since the amino acids in central channel of the binding sites for THs are very conserved. The possible influence on binding affinity therefore was focused on the N-terminus because of their location in molecule, which are situated around each entrance to the central channel where two binding sites for THs exist. Taken all observations together it has been proposed that the length and hydropathy of the N-terminus are important for THs binding of TTR (Prapunpoj *et al*, 2002).

The only X-ray crystal structure demonstrating the electron density for the N- and C-terminal regions is that of Hamilton *et al*., 1993, and there have not been any direct

structural analyses of the interaction of TTR N-terminal region with T_3 or T_4 directly. However, there are several indications demonstrated that THs interact with the N-terminal regions of TTR such as evidence that *N*-bromoacetyl-L-T_4 interacts with the Gly1 and Lys15 of human TTR (Cheng *et al*., 1977) and the Gly6Ser (glycine at position 6 was changed to serine) mutant of human TTR has a higher affinity for T_4 than wild type TTR (Fitcg *et al*., 1991). By using the chimeric TTRs should directly determine the effect of this region on binding affinity to THs of TTR.

In this thesis, the binding affinities to T_3 and T_4 of two chimeric TTRs, human/crocTTR and truncated crocTTR, were determined. The result clearly indicated the influence of the N-terminal region of the TTR subunits on accessibility of THs to the binding site as well as the strength and binding preference of THs to TTR. However, since the truncated crocTTR (lacking the N-terminal segment) had a K_d T_3/K_d T_4 ratio of 1, this indicated that TTR bound both T_3 and T_4 with the same strength and preference in the absence of the N-terminal segment. Comparison with the data that summarized the K_d value of all the recombinant TTRs studied (Table 3.2) demonstrated that crocTTR and its chimera in which N-terminal region was altered to that of human TTR (hu/crocTTR) had similar TH preference in binding. Both TTRs had higher affinity for T_3 than for T_4. However, in consideration only K_d for binding to T_4, hu/crocTTR had the binding affinity more similar to that of human TTR than crocTTR. These should imply that the core structure of TTR molecule plays a major role in determining of TH preferences (i.e. T_3 in case of crocTTR, and the N-terminal region provides an effect on strength or facilitate the accessibility of THs to the binding sites (i.e. T_4 in case of hu/crocTTR). To resolve this more precisely, further chimeric TTRs are required to be analyzed.

Table 4.1 The dissociation constant (K_d) for T_3 and T_4 of TTRs from vertebrate species

(From Chang *et al*., 1999 and Prapunpoj *et al*., 2000a, b, 2002; for review see Richardson, 2007)

Source of TTR	K_d T_4 (nM)	K_d T_3 (nM)	K_d T_4/$K_d$$T_3$
Eutherians			
Human	13.6	56.6	4.2
Sheep	11.3	63.5	3.2
Rat	8.0	67.2	8.4
Marsupials			
Wombat	21.8	97.8	4.5
Possum	15.9	206.1	12.9
Wallaby	13.8	65.3	4.7
Birds			
Emu	37.4	18.9	0.51
Chicken	28.8	12.3	0.43
Pigeon	25.3	16.1	0.64
Reptilians			
Crocodile	36.7	7.56	0.21
Amphibians			
Toad	508.0	248	0.49

3. Influence of N- and C-terminal regions on the binding of TTR to human RBP

Besides functions as a transporter of THs, TTR also acts as a carrier of vitamin A through the binding to retinol binding protein or RBP. In this thesis, influences of N- and C-terminal regions on the binding of TTR to RBP were studies using the recombinant wild type and chimeric TTRs as a tool. The western blot analysis was developed in order to determine the binding affinity between TTRs and RBP. By using this method, free and bound RBP were determined and used to quantify the K_d value using Scatchard analysis. The recombinant *C. porosus* TTR and all its chimeras had ability to bind to RBP. This revealed to the crossed interaction between TTR and RBP from different vertebrates consisting to the previous finding of the heterologous complex of human TTR-chicken RBP and human TTR-trout RBP (Kopelman *et al*., 1976 and Berni *et al*., 1992). The affinity constant (K_d) of human TTR to human RBP obtained in this study slightly different but still in range of 10^{-6} to 10^{-7} M as previous reported (Noy *et al*., 1992). These differences in K_d values might be due to conditions including pH, ionic strength and the methods used.

The binding affinity of TTR to RBP studied in this thesis varied among species. The binding between *C. porosus* TTR and human RBP (K_d was 2.54±0.23 µM) was different from that between human TTR and human RBP (K_d was 17.08±3.25 µM). It indicated that recombinant crocTTR bound human RBP with higher affinity than human TTR. TTR and RBP contribute 21 amino acids from each protein to the protein-protein recognition interface and most of them are in the C-terminal region (Nayler and Newcomer, 1999). There are 38 amino acids differences in the primary structure of human TTR and crocTTR. Amongst, only three residues are in the region of inter-protein contacts, i.e. the residues at position of 76, 84 and 114. While Lys, Ile and Tyr are the amino acid residue 76, 84 and 114 in human TTR, respectively, Ser, Leu and Phe are the residues 76, 84 and 114 in crocTTR, respectively. Point mutation of the amino acid residue Ile84 to Ser and Tyr114 to Cys in human TTR was reported leading to an occurrence of amyloidosis (Benson *et al*., 1983 and Ueno *et al*., 1990). The interference of mutation to the contact between TTR and RBP was only reported in Ile84 (Benson *et al*., 1983; Ueno *et al*., 1990; for review see Monaco *et al*., 2000). The mutation of Ile 84 to Ser was shown decreases the binding affinity of TTR to RBP, and consequently bring to decreasing level of RBP in serum. The X-ray

crystallography revealed that there were two Ile84 contributed to the interface between RBP and the TTR monomers, indicated the involved Ile84 from different TTR dimers and explained to a dramatic effect of this single point mutation to the binding between these two proteins. In this study, changing from Ile84, as in human TTR, to Leu in *C. porosus* TTR seems to increase, rather than decrease, stability of the binding between these two proteins. It is known that the affinity between TTR and RBP enhances at high ionic strength but reduces at low ionic strength, suggested contribution of the hydrophobic area in the intermolecular contacts (Peterson, 1971).

In case of the variant TTR, the change from the hydrophobic amino acid Ile84 to the more hydrophilic amino acid Ser might involve in the decrease in the binding affinity between these two proteins. However, in this study, changing of Ile 84 in human TTR to Leu 84 in crocTTR had no effect on the binding affinity of the two proteins. This could simply explain by the similar molecular charge of these two amino acids, i.e. both are the non-polar amino acids. Similarly, both Lys 76 in human TTR and Ser 76 in crocTTR are polar side chain amino acids, no dramatic effect on the binding affinity to RBP of TTR is expected. In contrast, Tyr114 in human TTR and Phe114 in crocTTR are much different from each other. Changing to more polarity of the binding site and increase in binding to RBP of crocTTR could be expected. The result supported this hypothesis. The K_d for binding to RBP of human TTR was ~7 times greater than that of crocTTR, indicated to higher binding affinity between crocTTR and human RBP. However, since the different was quite large, the influence from other factors such as the amino acid residues nearby the contacts and molecular folding of TTR could not eliminate.

Influence of hydropathy of the TTR N-terminal region on binding affinity to RBP was revealed in this thesis. It seems like that increase in hydrophilicity of the N-terminal region resulted in decrease of the binding affinity to RBP. This could be implied from the observation that while K_d for binding to human RBP of hu/crocTTR (shorter and more hydrophilic N-terminus in comparing to that of crocTTR) was 11.31±0.07 µM, which was ~5 times higher than the K_d of crocTTR, the K_d values of xeno/crocTTR (longer and more hydrophobic N-terminus in comparing to those of crocTTR and human TTR) and truncated crocTTR (shorter but less hydrophilic N-terminus in comparing to that of human TTR) did not alter from the wild type

crocTTR, i.e. the K_d was 1.66 ± 0.27 μM for xeno/crocTTR and 1.19±0.19 μM for truncated crocTTR. However, the binding affinity to human RBP of croc/huTTR was similar to that of human TTR purified from plasma even though hydropathy of the N-terminal region was changed to more hydrophobicity. Taken together, it could imply to greater influence of the hydrophilicity than hydrophobicity of the N-terminal region on the interaction between TTR and RBP.

In consideration of the influence of the C-terminal sequence, result revealed that the binding affinity of pigC/crocTTR (K_d was 2.34 ± 0.45 μM) to RBP was very similar to that of crocTTR, indicating that the C-terminal region might not directly involved to the binding interaction of TTR to RBP. Although most of amino acid residues that participated in the inter-contact of TTR-RBP complex situate at the C-terminal region of TTR (Naylor and Newcomer, 1999), only ten amino acid residues were changed in this study. In addition, these residues locate slightly far from those residues at the TTR-RBP contact.

Taken all together, it could hypothesize that the primary structure of the TTR N-terminal region has effect on binding of TTR to RBP. Hydropathy of the region possibly direct participates in interaction of residues at the contact or indirect influence on accessibility of RBP to the binding site. However, the C-terminal region, in comparing to the N-terminal region, has less or no influence on the binding. In addition, since during evolution of vertebrates from its ancestors similar to fish, amphibian and reptile, the N-terminal segment of TTR is shorter and more hydrophilic. It could also hypothesize that TTR lost ability to bind RBP during the evolution. To confirm this more precisely, further analysis of TTR-RBP binding using other chimeric TTRs are recommended.

4. Influence of N- and C-terminal regions on the protease property of TTR

There are several proteases that the proteolytic mechanism and substrate were well known nowadays. These enzymes are conventionally classified base on their catalytic mechanisms which can be divided into four canonical mechanistic classes i.e. the serine proteinases, cysteine proteinases, aspartic proteinases and metallo proteinases. All of which are involved in physiological and pathological mechanisms. Recently, a novel property of TTR has been identified. TTR has been described as a plasma protease that has apoA-I as one of specific substrate (Liz *et al*., 2004). However, as TTR lacks canonical structure for protease and its function apparently does not relate to proteolysis, TTR has been classified in the group of cryptic protease. The cryptic nature of TTR has received more increasing importance since then based on an idea that proteolytic activity can be hidden in the proteins with other described function (Liz and Sousa, 2005). TTR also has been revealed involve in cholesterol metabolism and the occurrence of amyloidosis, both as a trigger and inhibitor of the amyloid formation. Several studies paid more attentions to elucidate the involvement of this TTR ability to the physiological and pathological related functions, and some look into the structure of TTR to find out the relationship to this proteolysis property.

TTR has been believed having a non-canonical catalytic triad since there is no amino acid in the molecule lies in the position compatible to the canonical catalytic triad (i.e. His-Asp-Ser). However as an enzyme, one could expect that TTR should has a part that important for its proteolytic activity. Therefore, in this study, the influence of N- and C-terminal regions on this proteolytic activity of TTR has been conducted with the aim to reveal whether or not these regions, which shows influence on several properties of TTR including binding to THs and RBP, have an influence of the proteolytic activity of TTR. The insights obtained should not only describe importance of the regions but also reveal the relationship between changes in structure and function of TTR that occurred during evolution of vertebrates.

By using FITC-casein as a substrate, all examined TTRs except truncated crocTTR showed ability to hydrolyze this universal protease substrate. It revealed that TTR either from higher vertebrate, i.e. human, lower vertebrate, i.e. *C. porosus*, contained the proteolytic property. This finding was confirmed with using apoA-I as a substrate. By using this specific substrate all TTRs showed to have ability to cleave apoA-I. A

smaller fragment of apoA-I was generated in the reaction with TTR (Figure 3.20, 3.21), however in different manners. The recombinant human TTR had higher activity to apoA-I than that purified from plasma implying to a possible modification, which led to increase specificity and activity of the protein, occurred during post-translation of the TTR in *Pichia* cells. The previous study (Liz *et al*., 2004, 2007) revealed that TTR cleaved apoA-I at the C-terminus and this portion of apoA-I is important in lipid binding, self association and cholesterol efflux. The possibility of TTR to mediate C-terminal truncation of apoA-I that has an impact to lipid metabolism was, thus, postulated. In addition, an involvement of TTR to amyloigenesis of apoA-I was also assumed from the finding of co-deposition between mutated apoA-I fragment and wild-type TTR (Sousa *et al*., 2000). From this thesis, results showed that the cleaved apoA-I was continued degraded into smaller fragments if incubation was prolonged while no alteration was observed with the uncleaved fragment, implied to an increase susceptibility to be degraded of the cleaved fragment. This prompted us to a possible role of TTR in apoA-I clearance in addition to its previous proposed function as an apoA-I amyloid triggering (Liz *et al*., 2007).

Influence of N- and C-terminal sequences on the proteolytic activity of TTR was clearly demonstrated in this thesis. The activity altered when N- or C-terminal sequence was changed, though in different manners. The human TTR in which N-terminal sequence was changed to that of *C. porosus* TTR (croc/huTTR) had reduction in activity. In similar, the crocTTR that lacked N-terminus (truncated crocTTR) had no (with FITC-casein as substrate) or very low (with apoA-I as substrate) activity in comparing to the wild type crocTTR. In contrast, the activity extraordinary increase when the N-terminal region was changed to that of *X. laevis* TTR or the C-terminal region was changed to that of pig TTR. However, it showed that changing in the N-terminal region of crocTTR to that of human TTR did not affect this activity of TTR.

ApoA-I is a single polypeptide with a molecular mass of 28 kDa. It is a lipid binding protein, which act as a carrier protein, apoA-I turns the hydrophilic part to expose with water while keeps the hydrophobic part inside its molecule. The mechanism underlying and the amino acids those are involved in the binding interaction between TTR and apoA-I have not been elucidated. However, it was

demonstrated that binding of RBP interfered the proteolytic activity of TTR to apoA-I (Liz *et al*., 2004) suggesting that the substrate binding site for apoA-I may locate at or nearby the binding site for RBP. If this is true, N- and C-terminal regions should have influence on the binding of TTR to this protein. The results obtained confirmed this hypothesis, i.e. both N- and C-terminal regions showed influences on the cleavage ability of TTR. Changing the C-terminal sequence from shorter and more hydrophilic to longer and more hydrophobic resulted in higher catalytic activity of TTR. This is convincible that hydrophobic environment is important for binding and catalytic cleavage of TTR to apoA-I. The opposite influences of the N-terminal alteration from shorter and more hydrophilic to longer and more hydrophobic revealed in human TTR and crocTTR could suggest that length and composition of amino acids at the N-terminus could lead to different conformational change of the catalytic binding sites in TTR. This also indicated that not only the amino acid sequence at the N-terminus but also the amino acids nearby regulate the conformation of the catalytic site. Length and hydropathy of the N-terminal segment could either facilitate or interrupt the assessment of substrate to the catalytic site depending on the amino acid sequence nearby.

The results from this thesis could demonstrate the influence of N- and C-terminal regions on the proteolytic activity of TTR. However, it could not finally summarize to mechanism underlying and the relationship between the protease activity and change in the primary structure of the TTR subunit that occurred during evolution of vertebrates. More studies are needed. Construction of new chimeric TTRs in which both N- and C-terminal regions are altered to different or similar hydropathy and comparing their cleavage ability to other chimeric TTRs in which only N- or C-terminal region is changed are required. Revealing the three dimensional structure of the chimeric TTRs is also recommended as more insights of structure could be obtained.

The function of TTR in related to amyloidogenesis has been intensively studied just for recent and two opposite directional involvements has been demonstrated. One hand, TTR sequesters the Aβ resulted in decrease fibril formation and toxicity of the amyloid. On the other hand, TTR cleaves apoA-I and increase tendency to aggregate and form amyloid fibrils of the cleaved protein. These two functions are opposite but

depend on the same ability i.e. proteolysis of TTR. TTR is one of the negative acute phase plasma protein those their expression levels dramatically decrease following the acute phase response to trauma and inflammation. In contrast, TTR in the choroid plexus is not under negative acute phase regulation. Maintaining of TH to protect against hypothyroidism that might occur during trauma and inflammation, therefore, was proposed as one of functions of TTR in the brain. The cleavage of Aβ by TTR may be another function of TTR in brain. It seems like that TTR also contribute in clearance of the unwanted or toxic molecules via its proteolysis ability.

CHAPTER 5

CONCLUSIONS

The primary structure of protein is composed of different amino acid residues that are translated from gene. Number and sequence of amino acid control folding of protein into unique three dimensional structure and consequently control function of protein. The relationships between amino acid sequences and three dimensional structure, and between structure and function of the protein is the attractive work for researchers since knowledge can bring to the understanding of the protein's function and the change of structure during evolution of the protein that relate to its function. TTR is one of the most conserved protein in which the predominant change was only observed in the N-terminal region and lesser at the C-terminus. The change at these regions were proposed to involved to several functions of TTR, i.e. the thyroid hormones transporter, the retinol carrier and recently, the protease property. In order to elucidate the influence of theses regions to the functions of TTR, the recombinant chimeric TTRs in which the N- or C-terminal region is changed to the other TTR species were synthesis, and used as a tool. Several experiments that were performed can be summarized as followed.

1. Expression of the recombinant TTRs in yeast *P. pastoris*

Seven types of the recombinant TTRs were produced in this thesis using the heterologous protein expression system of *P. pastoris*, a methylotrophic yeast. Among these TTRs, five were from the new constructed clones (recombinant huTTR, croc/huTTR, hu/crocTTR, pigC/crocTTR and truncated crocTTR) and the other two were available clones in laboratory (crocTTR and xeno/crocTTR).

1.1 For the new constructed clones, the cDNAs for TTRs were successfully constructed in the pPIC3.5 (for recombinant huTTR) and in pPIC9 (for the other TTRs). The cDNA was expressed in *P. pastoris* and the TTRs were synthesized and extracellularly secreted to culture medium.

1.2 All the recombinant TTRs had the electrophoretic mobility under native condition faster than albumin in human serum, except truncated crocTTR and pigC/crocTTR migrated slightly slower than the other chimeric TTRs.

1.3 The subunit mass of the recombinant TTRs were 15.1, 15.5, 15.5 and 15.1 for crocTTR, hu/crocTTR, xeno/crocTTR and pigC/crocTTR, respectively. Three major bands were sometimes observed in truncated crocTTR with the masses of 16.6, 15.5 (main subunit) and 15.1 kDa, respectively. For the recombinant huTTR and croc/huTTR, the subunit masses obtained were 17.8 and 17.0, respectively.

1.4 By HPLC on a gel permeation column, the molecular weight of crocTTR, hu/crocTTR, xeno/crocTTR, pigC/crocTTR and truncated crocTTR were 60, 63, 66, 54 and 51 kDa, respectively, whereas the recombinant huTTR and its chimera, croc/huTTR, had the same molecular weight i.e. 63 kDa.

1.5 By Western blot analysis, the recombinant huTTR and croc/huTTR showed a specific reactivity with the antibody against human TTR purified from serum, whereas that of *C. porosus* TTR and its chimeras specifically reacted with the antiserum raised against recombinant crocTTR.

1.6 By N-terminal sequencing, the human TTR and truncated crocTTR showed complete N-terminal regions, whereas two additional amino acid residues were detected at the N-terminus of hu/crocTTR.

2. The influence of the N-terminal region on the binding of TTR to THs

Three types of recombinant TTRs were used as a tool to demonstrate the influence of the N-terminal region of TTR on the accession of THs to the TH binding sites of TTR. The recombinant huTTR had higher affinity for T_4 than for T_3 with the K_d T_3/K_d T_4 ratio of 2.70. The recombinant chimeric hu/crocTTR had higher affinity for T_3 than for T_4 with the K_d T_3/K_d T_4 ratio of 0.24, but the K_d values for T_3 of truncated crocTTR was similar to the K_d for T_3 of huTTR. This value was also similar to the K_d for T_4 of truncated crocTTR leading to a K_d T_3/K_d T_4 ratio of the truncated crocTTR to 0.97. Since the K_d for T_3 of hu/crocTTR was not significantly different form that of crocTTR (Prapunpoj *et al.*, 2002) while the ratio of K_d T_3/K_d T_4 was greater, it suggested that the N-terminal region exert a greater influence on the affinity of T_4

than on the affinity for T_3 and it also implied that the core of TTR had major influence in determining affinity for T_3 than for T_4.

3. The influence of N- and C-terminal regions on the binding of TTR to RBP

In order to elucidate the effect of N- and C-terminal regions on the binding of TTR to RBP, the recombinant crocTTR and its chimeras as well as the recombinant croc/huTTR were analyzed for their binding affinities to RBP and compared to that of human TTR purified from plasma. The recombinant crocTTR and its chimeras had higher affinity for RBP than human TTR and the chimeric croc/huTTR. The hu/crocTTR had lower affinity to RBP than the wild type crocTTR and the other chimeras originated from crocTTR (xeno/crocTTR, pigC/crocTTR and truncated crocTTR). The huTTR and croc/huTTR showed similar binding preference to RBP. These results led to a summary that the N-terminal region had effect on the interaction between TTR and RBP, and the hydrophilic N-terminus had greater influence than the hydrophobic N-terminus. In addition, it demonstrated that the C-terminal region of TTR might not be involved in the contact between TTR and RBP.

4. The influence of N- and C-terminal regions on protease activity of TTR

The N- and C-terminal regions of TTR showed an influence on the catalytic activity of TTR either by the analysis using FITC-casein or apoA-I as a substrate. The TTR that the N- or C-terminal region was changed in sequence showed difference in the catalytic activity in comparing to the wild type TTR either from human or *C. porosus*. The human TTR that the N-terminus was changed to that of *C. porosus* TTR had reduction of the catalytic activity in comparing to the wild type human TTR. The *C. porosus* TTR that lacked the N-terminus could not retain it activity. In addition, when the N-terminal and C-terminal regions of *C. porosus* TTR was altered to that of X. laevis TTR and pig TTR, respectively, the catalytic activity increased significantly. However, when the N-terminus of *C. porosus* TTR was changed to that of human TTR, the activity was not altered. This demonstrated that length and hydropathy of N- and C-termini influenced on changes in conformation of TTR and this led to change in catalytic activity of TTR either in promote or inhibit manner. Moreover, one possible novel function of TTR in clearing of apoA-I was postulated.

The study in this thesis could indicate to influence of N and C-terminal regions on the functions of TTR including binding to THs and RBP and proteolysis property of TTR. However, it could not be summarized to the mechanism underlying, and more studies are required.

For the binding of TTR to THs, comparing binding affinity of the other recombinant chimeric TTRs, i.e. the croc/huTTR, the pigC/croc TTR as well as the recombinant human TTR without the N-terminal region to THs are recommended to confirm the results obtained from this thesis.

For the binding of TTR to RBP, the other recombinant chimeric TTRs such as the recombinant crocodile TTR with the N-terminal region of other TTR that is shorter and more hydrophilic (TTR from other mammals) should be produced and binding affinity of these TTRs to RBP should be determined in order to confirm that hydrophilicity rather than hydrophobicity of the N-terminal region has greater influence on the binding interaction of TTR to RBP.

Also the other recombinant chimeric TTRs that originated from either human and crocodile TTR in which both N- and C-terminal regions are altered to different or similar hydropathy should be constructed and their cleavage ability in comparing to those of other chimeric TTRs in which only N- or C-terminal region is changed should be determined. In addition the catalytic activity of these chimeric TTRs should be examined in the presence of other specific substrates including amyloid β peptide and amidated neuropeptide Y (NPY), and serine protease inhibitors therefore more insight on the influence of the N and C-terminal region of TTR on this property as well as on the mechanism underlying of the proteolytic activity of TTR could be revealed.

REFERENCES

Achen, M.G., Duan, W., Pettersson, T.M., Harms, P.J., Richardson, S.J., Lawrence, M.C., Wettenhall, R.E., Aldred, A.R. and Schreiber, G. 1993. Transthyretin gene expression in choroid plexus first evolved in reptiles. Am. J. Physiol. 265, 982-989.

Aldred, A.R., Prapunpoj, P. and Schreiber, G. 1997. Evolution of shorter and more hydrophilic transthyretin N-termini by stepwise conversion of exon 2 into intron 1 sequences (shifting the 3' splice site of intron 1). Eur. J. Biochem. 246, 401-409.

Aleshire, S.L., Bradley, C.A., Richardson, L.D. and Parl, F.F. 1983. Localization of human Prealbumin in choroids plexus epithelium. J. Histochem. Biotechnol. 22, 151-168.

Armen R.S., Alonso D.O.V., Daggett V. 2004. Anatomy of an amyloidogenic intermediate: Conversion of β-sheet to α-sheet structure in transthyretin at acidic pH. Med. Chem. 12, 1847-63.

Barr, K.A., Hopkins, S.A. and Sreekrishna, K. 1992. Protocol for efficient secretion of HAS deledoped from *Pichia pastoris*. Pharm. Eng. 12, 48-51.

Bei, R., Schlom, J. and Kashmiri, S.V. 1995. Bacurovirus expression of a functional single chain immunoglobulin and its IL-2 fusion protein. J. Immunol. Methods 186, 245-255.

Bellovino, D., Morimoto, T., Tosetti, F. and Gaetani, S. 1996. Retinol binding protein and transthyretin are secreted as a complex formed in the endoplasmic reticulum in HepG2 human hepatocarcinoma cells. Exp. Cell Res. 222, 77-83.

Bellovino, D., Morimoto, T., Pisaniello, A. and Gaetani, S. 1998. *In vitro* and *in vivo* studies on transthyretin oligomerization. Exp. Cell Res. 243, 101-112.

Benson, M.D. and Dwulet, F.E. 1983. Prealbumin and retinol-binding protein serum concentration in the Indiana type hereditary amyloidosis. Arthritis Rheum. 26, 1493-1498.

Benvenga, S., Bartalena, L., Antolelli, A., Li Calzi, L., Di Pasquale, G. and Trimarchi, F. 1986. Radioimmunoassay for human thyroxine-binding prealbumin. Ann. Clin. Lab. Sci. 16, 231-240.

Berni R., Stoppini M. and Zapponi, M.C., 1992. The piscine plasma retinol-binding protein. Purification, partial amino acid sequence and interaction with the mammalian transthyretin of rainbow trout (*Oncorhynchus mykiss*)-retinol binding protein. Eur.J. Biochem. 204. 99-106.

Berni, R., Malpeli, G., Folli, C., Murrell, J. R., Liepnieks, J.J. and Benson, M.D. 1994. The Ile-84 to Ser amino acid substitution in transthyretin interferes with the interaction with plasma retinol-binding protein. J. Biol. Chem. 269, 23395-23398.

Bianco, A.C., Salvatore, D. and Gereben, B. 2002. Cellular and molecular biology and physiological role of the iodotyronine selenodeiodinases. Endocr. Rev. 23, 38-89.

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acid. Res. 7, 1513-1523.

Blake, C.C, Geisow, M.J, Swan, I.D, Rerat, C. and Rerat, B. 1974. Structure of human plasma prealbumin at 2.5 Å resolution. A preliminary report on the polypeptide chain conformation, quaternary structure and thyroxine binding. J. Mol. Biol. 88, 1-12.

Blake, C.C.F. and Oatley, S.J. 1977. Protein-DNA and protein-hormone interactions in prealbumin: a model of the thyroid hormone nuclear receptor?. Nature 268, 115-120.

Blake, C.C.F., Geisow, M.J., Oatley, S.J., Rerat, B. and Rerat, C. 1978. Structure of prealbumin: secondary, tertiary and quaternary interactions determined by fourier refinement at 1.8Å. J. Mol. Biol. 121, 339-356.

Blake, C.C.F. 1981. Prealbumin and the thyroid hormone receptor. Proc. Royal Soc. (Lond.). B211, 413-431.

Blay, P., Nillson, C., Hansson, S., Owman, C., Aldred, A.R. and Schreiber, G. 1993. An in vivo study of the effect of 5-HT and symphatetic nerves on transferin and transthyretin mRNA expression in rat choroids plexus and meninges. Brain Res. 662, 148-154.

Blomhoff, R. 1994. In vitamin A in Health and Disease, Marcel Dekker, New York, 1-35.

Brack, C.M., Duan, W., Hulbert, A.J. and Schreiber, G. 1995. Wallaby transthyretin. Comp. Biochem. Physiol. 110B, 523-529.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brake, A.J., Merryweather, J.P., Coit, D.G. Heberlein, U.A., Masiarz, F.R., Mullembach, G.T., Urdea, M.S., Valenzuela, P. and Barr, P.J. 1984. Alpha-factor-directed synthesis and secretion of mature foreign proteins in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 81, 4642-4646.

Branch, W. T. Jr., Robbins, J. and Edelhoch, H. 1971. Thyroxine-binding prealbumin: comformation in aqueous solution. J. Biol. Chem. 246, 6022-6018.

Branch, W.T., Robbins, J. and Edelhoch, H. 1972. Thyroxine-binding prealbumin. conformation in urea and guanidine. Arch. Biochem. Biophys. 152, 144-151.

Brent, G.A. 1994. The molecular basis of thyroid hormone action. New Eng. J. Med. 331, 847-853.

Brierley, R.A. 1998. Secretion of recombinant human insulin-like growth factorI (IGF-1). Methods Mol. Biol. 103, 149-177.

Cavallaro, T., Martone, R.L., Dwork, A.J., Schon, E.A. and Herbert, J. 1990. The retinal pigment epithelium is the unique site of transthyretin synthesis in the rat eye. Invest. Ophthalmol. Vis. Sci. 31, 497-501.

Cereghino, J.L. and Cregg, J.M. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 45-66.

Chang, L., Munro, S.L.A., Richardson, S.J. and Schreiber, G. 1999. Evolution of thyroid hormoe binding by transthyretin in birds and mammals. Eur. J. Biochem. 259, 534-542.

Chanoine, J.P., Alex, S., Fang, S.L., Stone, S., Leonard, J.L., Köhrle, J. and Braverman, L.E. 1992. Role of transthyretin in the transport of thyroxine from the blood to the choroids plexus, the cerebrospinal fluid, and the brain. Endocrinology 130, 933-938.

Cheng, S., Pages, R.A.,Saroff, H.A., Edelhoch, H. and Robbins, J. 1977. Analysis of thyroid hormone binding to human serum prealbumin by 8-anilinonapthalene-1-sulfonate fluorescence. Biochemistry 16, 3707-3713.

Chopra, I.J. 1976. An assessment of daily turnover and significance of thyroidal secretion of reverse T3. J. Clin. Invest.58, 32-40.

Chopra, I.J. 1996. Nature, Source, and Relative Significance of Circulating Thyroid Hormones. In: Werner and Ingbar's The Thyroid, A Fundamental and Clinical Text, 7th Ed. Braverman, L.E. and Utiger, R.D., Eds. Lippincott-Raven, Philadelphia, 112.

Claffey, K.P., Senger, D.R. and Spiegelman B.M. 1995. Structural requirements for dimerization, glycosylation, secretion, and biological function of VPF/VEGF. Biochim. Biophys. Acta 1246, 1-9.

Clare, J.J., Romanos, M.A., Rayment, F.B., Rowedder, J.E., Smith, M.A., Payne, M.M., Sreekrishna, K. and Henwood, C.A. 1991. Production of mouse epidermal growth factor in yeast: High-level secretion using *Pichia pastoris* strains containing multiple gene copies. Gene 105, 205-12.

Cohen, S.N., Chang, A.C. and Hsu, L. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U S A. 69, 2110-2114.

Couderc, R. and Baratti, J. 1980. Oxidation of methanol by the yeast *Pichia pastoris*: purification and properties of alcohol oxidase. Agric. Biol. Chem. 44, 2279-2289.

Costa, R. Goncalves, A., Saraiva, M.J. and Cardoso, I. 2008. Transthyretin binding to A-Beta peptide-Impact on A-Beta fibrillogenesis and toxicity. FEBS Letters 582, 936-942.

Craik, D.J., Duggan, B.M., and Munro, S.L.A. 1996. Conformations and Binding Interactions of Thyroid Hormone Analogues. In: Biological Inhibitors, Studies in Medicinal Chemistry, 2nd Ed. Choudhary, M.I., Ed. Harwood Academic Publishers, Amsterdam.

Cregg, J.M., Barringer, K.J., Hessler, A.Y. and Madden, K.R. 1985. *Pichia pastoris* as a host system for transformations. Mol. Cell Biol. 5, 3376-3385.

Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G. and Stillman, C.A. 1989. Functional characterization of the two alcohol oxidase genes from the yeast, *Pichia pastoris*. J. Mol. Cell Biol. 9, 1316-1323.

Cregg, J.M., Vedvick, T.S. and Raschke, W.C. 1993. Recent advances in the expression of foreign gene in *Pichia pastoris*. Bio/Technology 11, 905-910.

Cregg, J.M., Cereghino J.L., Shi, J. and Higgins, D.R. 2000. Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol. 16, 23-52.

De La Paz, P., Burridge, J.M., Oatley, S.J. and Blake, C.C.F. 1992. Multiple Modes of Binding of Thyroid Hormones and Other Iodothyronines to Human Plasma Transthyretin, In: The Design of Drugs to Macromolecular Targets. Beddell, C.R., Ed. Wiley, Chichester, 119-172.

Dickson, P.W., Howlett, G.J. and Schreiber, G. 1982. Metabolism of prealbumin in rats and changes induced by acute inflammation. Eur. J. Biochem. 129, 289-293.

Dickson, P.W., Howelett, G.J. and Schreiber, G. 1985. Rat transthyretin (prealbumin): molecular cloning, nucleotide sequence, and gene expression in liver and brain. J. Biol. Chem. 260, 8214-8219.

Dickson, P.W., Aldred, A.R., Marley, P.D., Tu, G.F., Howlett, G.J. and Schreiber, G. 1985. High prealbumin and transferring messenger RNA levels in the choroids plexus of rat brain. Biochem. Biophys. Res. Commun. 127, 890-895.

Dickson, P.W. and Schreiber, G. 1986. High levels of mRNA for transthyretin (prealbumin) in human choroids plexus. Neurosci Lett. 66, 311-315.

Dickson, P.W., Aldred, A.R., Marley, D., Bannister, D. and Schreiber, G. 1986. Rat choroids plexus specialize in the synthesis and the secretion of transthyretin (prealbumin) regulation of transthyretin synthesis in choroid plexus is independent from that in liver. J. Biol. Chem. 261, 3475-3478.

Dickson, P.W., Aldred, A.R., Menting, J.G.T., Marley, P.D., Sawyer, W.D. and Schreiber, G. 1987b. Thyroxine transport in choroids plexus. J. Biol. Chem. 262, 13907-13915.

Dodson, G. and Wlodawer, A. 1998. Catalytic triads and their relatives. Trends Biochem. Sci. 23, 347-352.

Duan, W., Cole, T. and Schreiber, G. 1989. Cloning and nucleotide sequencing of transthyretin (prealbumin) cDNA from rat choroids plexus and liver. Nucleic Acids Res. 17. 3979.

Duan, W., Achen, M.G., Richardson, S.J., Lawrence, M.C., Wettenhall, R.E.H., Jaworowski, A. and Schreiber, G. 1991. Isolation, characterization, cDNA cloning and gene expression of an avian transhyretin. Implications for the evolution of the structure and function of transthyretin in vertebrates. Eur. J. Biochem. 200, 679-687.

Duan, W., Richardson, S.J., Babon, J.J., Heyes, R.J., Southwell, B.R., Harms, P.J., Wettenhall, R.E.H., Dziegielewska, K.M., Selwood, L., Bradley, A.J., Brack, C. M. and Schreiber, G. 1995a. Evolution of transthyretin in marsupials. Eur. J. Biochem. 227, 396-406.

Duan, W., Richardson, S.J., Köhrle, J., Chang, L., Southwell, B.R., Harms, P.J., Brack, C.M., Pettersson, T.M. and Schreiber, G. 1995b. Binding of thyroxine to pig transthyretin, its cDNA structure, and other properties. Eur. J. Biochem. 230, 977-986.

Eales, J.G. 1984. The peripheral metabolism of thyroid hormone and regulation of thyroidal status in poikiotherms. J. Zool. 63, 1217-1231.

Ellis, S.B., Brust, P.F., Koutz, P.J., Waters, A.F., Harpold, M.M. and Gingeras, T.R. 1985. Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. Mol. Cell Biol. 5, 1111-1121.

Eneqvist, T., Lundberg, E., Nilsson, L., Abagyan, R. and Sauer-Eriksson. 2004.. The transthyretin-related protein family. Eur J. Biochem. 270, 518-532.

Farer, L.S., Robbins, J. and Blumberg, B.S. 1962. Electrophoresis of thyroxine in protein-free solutions and in sera deficient in binding proteins. Endocrinology 70, 679-85.

Ferguson, R.N., Edelhoch, H., Saroff, N.A. and Robbins, J. 1975. Negative cooperativity in the binding of thyroxine to human serum prealbumin. Biochemistry 14, 282-289.

Fitch, N.J.S., Akbari, M.T. and Ramsden, D.B. 1991. An inherited non amyloidogenic transthyretin variant, [Ser6]-TTR, with increased thyroxine-binding affinity, characterized by DNA sequencing. J. Endocrinol. 129, 309-313.

Furuya, H., Nakazato, M., Saraiva, M.J., Costa, S. P., Sasaki, H., Matsuo, H., Goto, I. and Sakaki, Y. 1989. Tetramer formation of a variant type human transthyretin (prealbumin) produced by *Escherichia coli* expression system. Biochem. Biophys. Res. Commun. 163, 851-859.

Foss T.R., Kelker M.S., Wiseman R.L., Wilson I.A. and Kelly J.W. 2005. Kinetic stabilization of the native state by protein engineering: implications for inhibition of transthyretin amyloidogenesis, J. Mol. Biol. 347, 841–54.

Gavrilesco, K., Courcon, J., Hillion, O., Uriel, J., Lewin, J. and Graba, P.A. 1955. Study of normal human cerebrospinal fluid by the immunoelectrophoretic method. Nature 176, 976.

Ghiso, J. and Frangione, B. 2002. Amyloidosis and Alzheimer's disease. Advance Drug Delivery Reviews 54, 1539-1551.

Giuseppin, M.L.F., van Eijk, H.M.J., Verduyn, C., Bante, I. and van Dijken, J.P. 1988. Production of catalase-free alcohol oxidase by *Hansenula polymorpha*. Applied. Microbiol. Biotechnology 28, 14-19.

Glass, C.K. and Holloway, J.M. 1990. Regulation of gene expression by the thyroid hormone receptor. Biochim. Biophis. Acta 1032, 157-176.

Golabek, A., Marques, M.A., Lalowski, M. and Wisniewski, T. 1995. Amyloid beta binding proteins *in vitro* and in normal human cerebrospinal fluid. Neurosci. Lett. 191, 79-82.

Goochee, C.F., Gramer, M.J. Anderson, D.C., Bahr, J.B. and Rasmussen, J.R. 1991. The oligosaccharides of glycoproteins bioprocess factors affecting the oligosaccharide structure and their effect on glycoprotein properties. Biotechnology 9, 1347-1355.

Goodman. D.S. and Raz, A. 1972. Extraction and recombinantion studies of the interaction of retinol with human plasma retinol-binding protein. J. Lipid Res. 13(3), 338-347.

Goodman, D.S. 1974. Vitamin A transport and retinol-binding protein metabolism. Vitam. Horm. 32, 167-180.

Goodman, D.S. 1984. Plasma-Retinol Binding Protein. In: The Retinoids. Sporn, M.B., Roberts, A.B. and Goodman, D.S., Eds. Academic Press, New York, 41-88.

Greenspan, F.S. 1994. In: Basic and Clinical Endocrinology. Greenspan, F.S. and Baxter, J.D., Eds. Appleton & Lange, Norwalk, Conn. 1, 160-226.

Grinna, L.S. and Tschopp, J.F. 1989. Size distribution and general structure features of N-linked oligosaccharides from the methylotrophic yeast *Pichia pastoris*. Yeast 5, 107-115.

Groner, A. 1986. Specificity and Safety of Baculoviruses. In: The Biology of Baculoviruses. Granados, R.R. and Federici, B.A., Eds. CRC Press, Boca raton, Florida, 177.

Gross, J., Pitt-Rivers., R. and Trotter, W.R. 1952. Effect of 3,5,3′-triiodothyronine in myxedema. Lancet. 24, 1044-1045.

Hamilton, J.A., Steinrauf, L.K., Braden, B.C., Liepnieks, B.J., Benson, M.D., Holmgren, G., Sandgren, O. and Steen, L. 1993. The X-ray crystal structure refinementso f normal human transthyretin and the amyloidogenic Val-30 → Met variant to 1.7-Å resolution. J. Biol. Chem. 268, 2416-2424.

Hamilton, J.A., and Benson, M.D. 2001. Transthyretin: a review from a structural perspective. Cell Mol. Life Sci. 58, 1491–1521.

Harington, C.R. and Rivers, R.V.P. 1945. The chemical conversion of di-iodotyrosine into thyroxine. Biochem. J. 39, 157-164.

Harms, P.J., Tu, G.F., Richardson, S.J., Aldred, A.R., Jaworoski, A. and Schreiber, G. 1991. Transthyretin (prealbumin) gene expression in choroids plexus is strongly conserved during evolution of vertebrates. Comp. Biochem. Physiol. 99, 239-249.

Hayashi, H., Ohmiya, Y. and Suzuki, S. 1991. Thyrosine-130 in bullfrog thyroglobulin is a thyroid hormone generating site. FEBS Lett. 292, 168-170.

Heller, J. and Horwitz, J. 1974. The binding stoichiometry of human plasma retinol-binding protein to prealbumin. J. Biol.Chem. 249, 5933-5938.

Hennebry, S.C., Wright, H.M., Likic, V. and Richardson, S.J. 2006a. Structural and functional evolution of transthyretin and transthyretin-like proteins. Proteins 64, 1024-1045.

Hennebry, S.C., Buckle, A.M., Law, R.H., Richardson, S.J. and Whisstock, J.C. 2006b. The crystal structure of the transthyretin-like protein from Salmonella

Dublin reveals the structural basis for 5-hydroxyyisoorate hydrolase activity. J. Mol. Biol. 359, 1389-1399.

Herbert, J., Wilcox, J.N., Pham, K.T.C., Fremeau, R.T., Zeviani, M.Jr., Dwork, A., Soprano, D.R., Makover, A., Goodman, D.S., Zimmerman, E.A., Roberts, J.L. and Schon, E.A. 1986. Transthyretin: a choroids plexus-specific transport protein in human brain. Neurology 36, 900-911.

Higgins, D.R. and Cregg, J.M. 1998. Introduction to *Pichia pastoris*. In: Methods in Molecular Biology: *Pichia* Protocols. Higgins, D.R. and Cregg, J.M., Eds. Human Press, Totowa, New Jersey. 103, 1-15.

Higuchi, M., Iwata, N. and Saido, T.C. 2005. Understanding molecular mechanisms of proteolysis in alzheimer's disease: progress toward therapeutic interventions. Biochim. Biophys. Acta 1751, 60-67.

Hillier, A.P. 1970. The binding of thyroid hormones to phospohlipid membranes. J. Physiol-London 211, 585-597.

Hörnberg, A. 2004. Transthyretin from a structural perspective. ISBN. 91, 7305-7593.

Hourdry, J. 1993. Passage to the terrestrial life in amphibians. II. Endocrine determinism. Zoo. Sci. 10, 887-902.

Imamura, S., Mori, Y., Murata, Y., Yamamori, I., Miura, Y., Oiso, Y. Seo, H., Matsui, N. and Refetoff, S. 1991. Molecular cloning and primary structure of rat transthyrtin binding globulin. Biochemistry 30, 5406-5411.

Ingbar, S.H. 1958. Pre-albumin: a thyroxine-binding protein of human plasma. Endocrinology 63, 256-259

Irikura, D., Yanaoka, Y. and Urade, Y. 1997. Bovine TTR sequence submitted to EMBL/Genbank/DDBJ databases, December 1997. See http://www.expasy.org/uniprot/046375 (accessed November 2006).

Jaarsveld, P.P.V., Edelhoc, H., Goodman, D.S. and Robbins, J. 1973. The interaction of human plasma retinol-binding protein and prealbumin. J. Biol. Chem. 248, 4698-4705.

Jacobsson, B. 1988. *In situ* localization of transthyretin mRNA in the adult human liver, choroid plexus and pancreatic islet and in endocrine tumors of the pancreas and gut. J. Histochem. Cell Biol. 91, 299-304.

Jacobson, D.R., Pastore, R.D., Yaghoubian, R., Kanel, I. Gallo, G., Buck, F.S. and Buxbaum, J.N. 1997. Variant-sequence transthyretin(isoleucine 122) in late-onset cardiac amyloidosis in black Americans. N. Engl. J. Med. 336(7), 466-473

Jaworowski, A., Fang, Z.P., Khong, T.F. and Augusteyn, R.C. 1995. Protein synthesis and secretion by cultured retinal pigment epithelia. Biochim. Biophys. Acta 1245, 121-129.

Jeang, K.T., Giam, C.Z., Nerenberg, M. and Khoury, G. 1987. Abundant synthesis of functional human T-cell leukemia virus type I $p40^{x}$ protein in eukaryotic cells by using a baculovirus expression vector. J. Virol. 61(3), 708-713.

Julius, D. Schekman, R. and Throner, J. 1984a. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. Cell 36, 309-818.

Julius, D., Brake, A., Blair, L., Kunisawa, R. and Thorner, J. 1984b. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. Cell 37, 1075-1089.

Kabat, E.A., Moore, D.H. and Landow, H. 1942b. An electrophoretic study of the protein components in cerebrospinal fluid and their relationship to the serum proteins. J. Clin. Invest. 21, 571-577

Kanai, M., Raz, A. and Goodman, D.S. 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. J. Clin. Invest. 47, 2025-2044.

Kanda, Y., Goodman, D.S., Canfield, R.E. and Morgan, F.J. 1974. The amino acid sequence of human plasma prealbumin. J. Biol. Chem. 249, 6796-6805.

Kato, M., Kato, K. and Goodman, D.S. 1984. Immunocytochemical studies on the localization of plasma and of cellular retinol-binding proteins and of transthyretin (prealbumin) in rat liver and kidney. J. Biol. Chem. 98, 1696-1704.

Kato, M., Kato, K., Blaner, W.S., Chertow, B.S. and Goodman, D.S. 1985. Plasma and cellular retinoid-binding proteins and transthyretin (prealbumin) are all localized in the islets of Langerhans in the rat. Proc. Natl. Acad. Sci. USA. 82, 2488-2492.

Katoh, M., Kanai, M., Kamako, M., Ohno, S., Fuijii, Y. and Nagata, T. 1982. localization of retinol-binding protein and prealbumin in the human kidney with

an unlabeled enzyme immunohistochemical method. Acta Histochem. Cytochem. 15, 68-75.

Köhrle, J, Spanka, M., Irmscher, K. and Hesch, R.D. 1988. Flavonoid Effects on Transport, Metabolismaction of Thyroid Hormones. In: Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medical Properties. Cody, V., Harborne, J.B. and Beretz, A., Eds. Alan R. Liss, New York, 323-340.

Köhrle, J. 2002. Iodothyronine deiodinases. Methods Enzymol. 347, 125-167.

Kopelman, M, Cogan, U., Mokady, S., Shinitzky, M. 1976. The interaction between retinol binding proteins and prealbumins atudied by fluorescence polarization. Biochim. Biophys. Acta 439, 449-460.

Koutz, P., Davis, G.R., Stillman, C., Barringer, K., Cregg, J. and Thill, G. 1989. Structural comparison of the *Pichia pastoris* alcohol oxidase genes. Yeast 5, 167-77.

Kronenberg, H.M., Melmed, S., Polonsky, K.S. and Larsen, P.R. Mechanism of Thyroid Hormone Action. 2008.

http://mdconsult.com/das/book/body/0/0/1555/I4-u (accessed 23/01/09), part of Kronenberg: Williams Textbook of Endocrinology. http://mdconsult.com (accessed 23/01/09).

Kurjan, J. and Herskowitz, I. 1982. Structure of yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. Cell 30, 933-943.

Landford, R.E. 1988. Expression of Simian virus 40 T antigen in insect cells using a baculovirus vector. Virology 167, 72-81.

Laemmli, U. and Favre, M. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. 80, 575-599.

Larsson, M., Pettersson, T. and Carlström, A. 1985. Thyroid hormone binding in serum of 15 vertebrate species. Isolation of thyroxine-binding globulin and pre-albumin analogs. Gen. Comp. Endocrinol. 58, 360-375.

Lerman, J. 1953. The physiologic activity of L- triiodothyronine. J. Clin. Endocrinol. Metab. 13, 1341-1346.

Link, C.D. 1995. Expression of human beta-amyloid peptide in transgenic *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U S A. 92, 9368–9372.

Liz, M.A., Faro, C.J., Saraiva, M.J. and Sousa, M.M. 2004. Transthyretin, a new cryptic protease. J. Biol. Chem. 279, 21431-21438.

Liz, M.A. and Sousa, M.M. 2005. Deciphering cryptic proteases. Cell Mol. Life Sci. 62, 989–1002.

Liz, M.A., Gomes, C.M., Saraiva, M.J. and Sousa, M.M. 2007. ApoA-I cleaved by transthyretin has reduced ability to promote cholesterol efflux and increase amyloidogenicity. J. Lipid Research, 48. 2385-2389.

Liz, M.A., Fleming, C.E., Nunes, A.F., Almeida, M.R., Mar, F.M., Choe, Y., Craik, C.S., Power, J.C., Bogyo, M. and Sousa, M.M. 2009. Substrate specificity of transthyretin: identification of natural substrates in the nervous system. Biochem. J. 419, 467-474.

Loughna, S., Benett, P. and Moore, G. 1995. Molecular analysis of the expression of transthyretin in intestine and liver from trisomy 18 fetuses. Hum. Genet. 95, 89-95.

Luckow, V.A. and Summer, M.D. 1988. Trends in the development of baculoviris expression systems. Bio/Technology 6, 47-55

Lundberg, E., Bäckstöm, S., Sauer, U.H. and Sauer-Eriksson, E. 2006. The transthyretin related protein: Structural investigation of a novel protein family. J. Struct. Biol. 155, 445-457.

Makover, A., Moriwaki, H., Ramakrishnan, R., Saraiva, M.J.M., Blaner, W.S. and Goodman, D.S. 1988. Plasma transthyretin: tissue sites of degradation and turnover in the rat. J. Biol. Chem. 263, 8598-8603.

Manzon, R.G., Neuls, T.M. and Manzon, L.A. 2007. Molecular cloning, tissue distribution, and developmental expression of lamprey transthyretins. Gen. Comp. Endocrinol. 151, 55-65.

Marston, F.A. and Hartley, D.L. 1990. Solubilization of protein aggregates. Methods Enzymol. 182, 264-76.

Matsubara, K., Mizuguchi, M. and Kawano, K. 2003. Expression of a synthetic gene encoding human transthyretin in *Escherichia coli*. Protein Expr. Purif. 30, 55-61.

Mendel, C.M., Weisiger, R.A., Jones, A.L., and Cavalieri, R.R. 1987. Thyroid hormone-binding proteins in plasma facilitate uniform distribution of thyroxine within tissues : a perfused rat liver study. Endocrinol. 120, 1742-1749.

Mendel, C.M., Cavalieri, R.R., Gavin, L.A., Pettersson, T. and Inoue, M. 1989. Thyroxine transport and distribution in Nagase analbuminemic rats. J.Clin Invest. 83, 143-148.

Miller, D.L., Papayannopolous, I.A., Styles, J., Bobin, S.A., Lin, Y.Y., Biemann, L.K. and Iqbal, K. 1993. PPeptides compositions of the cerebrovascular and senile plaque core amyloid deposits of Alaheimer's desease. Arch. Biochem. Biophys. 301, 41-52.

Miller, S.R., Sekijima, Y. and Kelly, J.W. 2004. Native state stabilization by NSAIDs inhibits transthyretin amyloidogenesis from the most common familial disease variants. Lab. Invest. 84. 545-552.

Miwa, S., Tamano, K. and Inui, Y. 1992. Thyroid hormone stimulates gastreic development in flounder larvae during metamorphosis. J. Exp. Zool. 261,, 424-430.

Miyamato, H., Larochelle, F.T., Suzuki, M., Freeman, M. and Frieden, E. 1977. Studies on thyroid hormones and their binding in bullfrog tadpole plasma during metamorphosis. Gen. Comp. Endocrinol.33, 254-266.

Miyamoto, C., Smith, G.E., Farrell-Towt, J., Chizzonite, R., Summer, M.D. and Ju, G. 1985. Production of human c-myc protein in insect cells infected with a baculovirus expression vector. Mol. Cell. Biol. 5, 2860.

Monaco, H.L., Mancia, F., Rizzi, M. and Coda, A. 1994. Crystallization of the macromolecular complex trasthyretin-retinol binding protein. J. Mol. Biol. 244, 110-113.

Monaco, H.L., Rizzi, M. and Coda, A. 1995. Structure of a complex of two plasma proteins: transthyretin and retinol bonding protein. Science 268, 1039-1041.

Monaco, H.L. 2000. The transtyretin-retinol-binding-protein complex. Biochim. Biophysic. Acta 1482, 65-72.

Montesino, R., Nimtz, M., Quintero, O., Garcia, R., Falcon, V. and Cremata, J.A. 1998. Characterization of the oligosaccharides assembled on the *Pichia pastoris* –express recombinant aspartic protease. Glycoprotein 9, 1037-1043.

Montecinos, H.A., Richter, H., Caprile, T. and Rodríguez, E.M. 2005. Synthesis of transthyretin by the ependymal cells of the subcommissural organ. Cell Tissue Res. 320, 487–499.

Morgado, I., Melo, E.P., Lundberg, E., Estrela, N.L., Sauer-Eriksson A.E. and Power, D.M. 2008. Hormone affinity and fibril formation of piscine transthyretin: The role of the N-terminal. Mol Cell Endrocrinol. 295 (1-2), 48-58.

Mori, H., Takio, K., Ogawara, M. and Selkoe, D.J. 1992. Mass spectrometry of purified amyloid protein in Alzheimer's disease. J. Biol. Chem. 24, 17082-17086.

Morrissey, J. 1981. Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117, 307-310.

Munro, S.L., Lim, C.F., Hall, J.G., Barlow, J.W., Craik, D.J., Topliss, D.J. and Stockigt, J.R. 1989. Drug competition for thyroxine binding to transthyretin(prealbumin): Comparison with effects on thyroxine-binding globulin. J. Clin. Endocrinol. Metab. 68, 1141-1147.

Murrell, J.R., Schoner, R.G., Liepnieks, J.J., Rosen, H.N., Moses, A.C. and Benson, M.D. 1992. Production and functional analysis of normal and variant recombinant human transthyretin proteins. J. Biol. Chem. 267, 16595-600.

Naylor, H.M. and Newcomer, M.E. 1999. The structure of human retinol-binding protein (RBP) with its carrier protein transthyretin reveals an interaction with the carboxy terminus of RBP. J. Biochem. 38, 2647-2653.

Neuman, P., Cody, V. and Wojtczak, A. 2001. Structural basis of negative cooperativity in transthyretin. Acta Biochimica Polonica 48, 867-875.

Newcomer, M.E., Jones, T.A., Åqvist, J., Sundelin, J., Eriksson, U., Rask, L. and Peterson, P.A. 1984. EMBO. J. 3, 1451-1454.

Nilsson, S.F., Rask, L. and Peterson, P. 1975. Studies on thyroid hormone-binding proteins: II. Binding of thyroid hormones, retinol-binding protein, and fluorescent probes to prealbumin and effects of thyroxine on prealbumin subunit self Association. J. Biol. Chem. 250, 8554-8563.

Noy, N. Slosberg, E. and Scarlata, S. 1992. Interactions of retinol with binding proteins: studies with retinol-binding protein and with transthyretin. Biochemistry 31, 11118-11124.

Nyunoya, H., Akagi, T., Ogura, T., Maeda, S. and Shimotohno, K. 1988. Evidence for phosphorylation of trans-activator p40x of human T-cell leukemia virus type I produced in insect cells with baculovirus expression vector. Virology 167, 538-544.

Oatley, S.J., Burridge, J.M. and Blake, C.C.F. 1982. X-ray Crystallographic Studies of Thyroid Hormone Analogue and Competitor Binding Interactions with Human Serum Prealbumin. In: Hormone Antagonists. Agarwal, M.K., Ed. Walter de Gruyter, Berlin. 705-715.

Oatley, S.J., Blaney, J.M., Langridge, R. and Kollman, P.A. 1984. Molecular-mechanical studies of hormone-protein interactions: the interaction of T_4 and T_3 with prealbumin. Biopolymers 23, 2931-2941.

Ohmiya, Y., Hayashi, H., Kondo, T. and Kondo, Y.1990. Location of dehydroalanine residues in the amino acid sequence of bovine thyroglobulin: identification of 'donor' thyrosine sites for hormonogenesis in thyroglobulin. J. Biol. Chem. 265, 9066-9071.

Orozco, A. Silva, J.E., Valverde, R.C. 1997. Rainboe trout liver expresss two iidothyronine phenolic ring deiodinase pathwaya with the characteristics of mammalian type I and II deiodinases. Endocrinology 138, 254-258.

Ouchterlony, O. 1956. Antigen-antibody reactions in gel. V Types of reactions in co-ordinated systems of diffusion. Acta. Path. Microbiol. Scand. 32, 231-240.

Pages, R.A., Robbins, J. and Edelhoch, H. 1973. Binding of thyroxine and thyroxine analogs to human serum prealbumin. Biochemistry 12, 2773-2779.

Palha, J.A., Nissanov, J., Fernandes, R., Sousa, J.C., Bertrand, L., Dratman, M.B., Morreal De Escobar, G., Gottesmanne, M. and Saraiva, M.J. 2002. Thyroid hormone distribution in mouse brain: the role of transthyretin. Neuroscience 113, 837-847.

Palumbo, G., Geltile, F., Condorelli, G.L. and Salvatore, G. 1990. The eariest site of iodination in thyroglobulin is residue no. 5. J. Biol. Chem. 265, 8887-8892.

Pardridge, W.M. 1987. Plasma protein-mediated transport of steroid and thyroid hormones. Am. J. Physiol. 252, E157-164.

Pardridge, W.M. and Mietus, J.S. 1980. Influx of thyroid hormones into rat liver *in vivo*. J. Clin. Invest. 66, 367-374.

Peterson, P.A. 1971. Studies on the interaction between prealbumin, retinol-binding protein and vitamin A. J. Biol. Chem. 246, 44-49.

Peterson, P.A. and Rask, L. 1971. Studies on the fluorescence of the human vitamin A-transporting plasma protein complex and its individual components. J. Biol. Chem. 246, 7544-50.

Pfeifer, T.A. 1998. Expression of heterologous proteins in stable insect cell culture. Curr. Opinion Biotech. 9, 518-521.

Pieter, P., Jaarsveld, V., Edelhoch, H., Goodman, S.D. and Robbins, J. 1973. The interaction of human plasma retinol-binding protein with prealbumin. J. Biol. Chem. 248, 4698-4705.

Power, D.M., Elias, N.P., Richardson, S.J., Mendes, J., Soares, C.M. and Santos, C.R.A. 2000. Evolution of the thyroid hormone- binding protein, Transthyretin. Gen. Comp. Endocrinol. 119, 241-255.

Prapunpoj, P., Leelawatwatana, L., Schreiber, G. and Richardson, S.J. 2006. Change in structure of the N-terminal region of transthyretin produces change in affinity of transthyretin to T_4 and T_3. FEBS Journal 273, 4013-4023.

Prapunpoj, P., Richardson, S.J., Fumagalli, L. and Schreiber, G. 2000a. The evolution of the thyroid hormone distributor protein transthyretin in the order insectivora, class mammalia. J. Mol. Biol. Evol. 17, 1199-1209.

Prapunpoj, P., Yamauchi, K., Nishiyama, N., Richardson, S. and Schreiber, G. 2000b. Evolution of structure, ontogeny of gene expression, and function of *Xenopus laevis* transthyretin. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, R2026-R2041.

Prapunpoj, P., Richardson, S.J. and Schreiber, G. 2002. Crocodile Transthyretin: structure, function, and evolution. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, 885-896.

Raghu P., Ravinder P. and Sivakumar B. 2003. A new method for purification of human plasma retinol-binding protein and transthyretin. Biotechnol. Appl. Biochem. 38, 19– 24.

Raghu, P. and Sivakumar, B. 2004. Interaction amongst plasma retinol-binding protein, transthyretin and their ligands: implications in vitamin A homeostasis and transthyretin amyloidosis. Biochim. Biophys. Acta 1730 (1), 1-9.

Raz, A. and Goodman, D.S. 1969. The interaction of thyroxine with human plasma prealbumin and with the prealbumin-retinol-binding protein complex. J. Biol. Chem. 244, 3230-3237.

Refetoff, S., Robin, N.I. and Fang, V.S. 1970. Parameters of thyroid function in serum of 16 selected vertebrate species. A study of PBI, serum T4, free T4, and the pattern of T_4 and T_3 binding to serum proteins. Endocrinology 86, 793-805.

Reiber, H. 1997. CSF Flow-Its Influence on CSF Concentrations of Brain-Derived and Blood-Derived Proteins. In: Neurochemistry. Teelken, A. and Korf, I., Eds. New York, Plenum. 51-72

Reixach, N. Foss, T.R. Sanelli, E. Pascual, J., Kelly, J.W. and Buxbaun, J.N. 2008. Human-murine transthyretin heterotetramers are kinetically stable and non-amyloidogenic: a lesson in the generation of transgenic models of diseases involving oligomeric proteins. J. Biol. Chem. 283, 2098-2107.

Richardson, S.J., Bradley, A.J., Duan, W., Wettenhall, R.E., Harms, P.J., Babon, J. J., Southwell, B.R., Nicol, S., Donnellan, S.C. and Schreiber, G. 1994. Evolution of marsupial and other vertebrate thyroxine-binding plasma proteins. Am. J. Physiol. 266, R1359-R1370.

Richardson, S.J., Wettenhall, R.E.H. and Schreiber, G. 1996. Evolution of transthyretin gene expression in the liver of *Didelphis virginiana* and other American marsupials. Endocrinology 137, 3507-3512.

Richardson, S.J., Hennebry, S.C., Smith, B.J. and Wright, H.M. 2005. Evolution of the thyroid hormone distributor protein transthyretin in microbes, *C. elegans*, and vertebrates. Ann. N.Y. Acad. Sci. 1040, 448-451.

Richardson, S.J. 2007. Cell and molecular biology of transthyretin and thyroid hormones. Inter. Rev. Cytology 258, 137-193.

Ridder, R., Schmitz, R., Legay, F. and Gram, H. 1995. Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*. Bio/Technology 13, 255-260.

Robbins, J. 1976. Thyroxine-binding proteins. Prog. Clin. Biol. Res. 5, 331-355.

Robbins, J. Cheng, S.Y., Gershengorn, M.C., Glinoer, D. Cahnmann, H.J. and Edelhoch, H. 1978. Thyroxine transport proteins of plasma: molecular properties and biosynthesis. Recent Progr. Horm. Res. 34, 477-519.

Rocher, A.E., Lowenson, J.D., Clrrke, S., Wolkow, C., Wang, R., Cotter, R.J. Reardon, I.M., Zuurcher-Neely, H.A., Heinrikson, R.L., Ball, M.J. and Greenberg, B.D. 1993. Structural alterations in the peptide backbone of a β-amyloid core protein may account for its deposition and stability in Alzheimer's desease. J. Biol. Chem. 268, 3072-3083.

Rosen, H.N., Murrell, J.R., Liepnieks, J.J., Benson, M.D., Cody, V. and Moses, A.C. 1994. Threonine for alanine substitution at position 109 of transthyretin differentially alters human transthyretin's affinity for iodothyronines. Endocrinology 134, 27-34.

Rostom, A.A., Margaret, S., Richardson, S.J., Schreiber, G., Jarvis, S., Bateman, R., Dobson, C.M. and Robinson, C.V. 1998. Dissection of multi protein complexes using mass spectrometry: subunit interactions in transthyretin and retinol-binding protein complexes. Proteins: Structure, Function and Genetics Supply. 2, 3-11.

Samuels, H.H., Forman, B.M., Horowitz, Z.D. and Ye, Z.S. 1988. Regulation of gene expression by thyroid hormone. J. Clin. Invest. 81, 957-967.

Samuels, H.H., Forman, B.M., Horowitz, Z.D. and Ye, Z.S. 1989. Regulation of gene expression by thyroid hormone. Annu. Rev. Physiol. 51, 623-639.

Sang, J.H. 1981. Drosophila cells and cell lines. Adv. Cell Culture 1, 125.

Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74(2), 5463-7.

Santos, C.R.A. and Power, D.M. 1999. Identification of transthyretin in fish (*Sparus aurata*): cDNA cloning and characterization. Endocrinology 140, 2430-2433.

Saraiva, M.J.M. 2002. Hereditary transthyretin amyloidosis: molecular basis and therapeutical strategies. Expert Review in Molecular Medicine, 1-11.

Sarver, N. and Stollar, V. 1977. Sindbis virus-induced cytopathic effect in clones of *Aedes albopictus* (Singh) cells. Virology 80, 390-400.

Sasaki, H., Yoshika, N., Takagi, Y. and Sasaki, Y. 1985. Structure of the chromosomal gene for human serum pralbumin. Gene 37, 191-197.

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. Annu. N.Y. Acad. Sci. 51, 660-672.

Schneider, I. 1972. Cell lines derived from late embryonic stages of Drosophila melanogaster. J. Embryol. Exp. Morphol. 27, 353-365.

Schreiber, G. and Howlett, G.J. 1983. Synthesis and Secretion of Acute-Phase Proteins. In: Plasma Protein Secretion by The Liver. Glaumann, H., Peters, T. and Redman, C., Eds. Academic Press, London, 423-449.

Schreiber, G., Aldred, A.R., Jaworowski, A., Nilsson, C., Achen, M.G. and Segal, M.B. 1990. Thyroxine transport from blood to brain via transthyretin synthesis in choroids plexus. Am. J. Physiol. 258, R338-R345.

Schieber, G and Richardson, S.J. 1997. The evolution of gene expression, structure and function of transthyretin. Comp. Biochem. Physiol. 116, 137-160.

Schreiber, G., Prapunpoj, P., Chang, L., Richardson, S.J., Aldred, A.R. and Munro, S.L. 1998. Evolution of thyroid hormone distribution. Clin. Exp. Pharmacol. Physiol. 25, 728-732.

Scheiber, G., Prapunpoj, P., Chang, L., Richardson, S.T., Aldred, R.A. and Munro, S.L. 1999. Evolution of thyroid hormone distribution. Clin. Exp. Pharmacol. Physiol. 25, 728-732.

Schreiber, G.. 2002. The evolutionary and integrative roles of transthyretin in thyroid hormone homeostasis. J. Endocrinology 175, 61-73.

Schwarzman, A.L., Gregori, L., Vitek, M.P., Lyubski, S., Strittmatter, W.J., Enghilde, J.J., Bhasin, R., Silverman, J., Weisgraber, K.H., Coyle, P.K., Zagorski, M.G., Talafous, J., Eisenberg, M., Saunders, A.M., Roses, A.D. and Goldgaber, D. 1994. Transthyretin sequesters amyloid β protein and prevents amyloid formation. PNAS. 91, 8368-8327.

Schwarzman, A.L. and Goldgaber, D. 1996. Interaction of transthyretin with amyloid beta protein: binding an dinhibition of amyloid formation. Ciba Found Symp 199, 146-160.

Scorer, C.A., Clare, J.J., McCombie, W.R., Romanos, M.A. and Sreekrishna, K. 1994. Rapid selection using G418 of high coppy number transformants of *Pichia pastoris* for high-level foreigh gene expression. Bio/Technology 12, 181-184.

Seibert, F.B. and Nelson, J.W. 1942. Electrophoretic study of the blood protein response in tuberculosis. J. Biol. Chem. 143, 29-38.

Selkoe, D.J. 2004. Alzheimer disease: mechanistic understanding pedicts novel therapies. Ann. Intern. Med. 140, 627-638.

Shambaugh, G.E. 1986. Thyroid Hormone Action. Biology and Cellular Effects. In: Werner's the Thyroid. Ingbar, S.H. and Braverman, L.E., Eds. Philadelphia, PA: Lippincoff , 201-218.

Shi, Y.B., Fu, L., Hsia, S.C., Tomita, A., and Buchholz, D. 2001. Thyroid hormone regulation of apoptotic tissue remodeling during anuran metamorphosis. Cell Res. 11, 245-252.

Sivaprasadarao, A. and Findlay, J.B.C. 1994. In Vitamin A in Health and Disease, Marcel Dekker, New York, 87-117.

Solovyov, K.V., Gasteva, A.A., Egorov, V.V., Aleinikova, T.D.., Sirotkin, A.K., Shavartsman, A.I. and Shavlovsky, M.M. 2006. Role of the C-terminal fragment of human transthyretin in abnormal fibrillogenesis. Biochemistry 71, 543-549.

Soprano, D.R., Soprano, K.J. and Goodman, D.S. 1986. Retinol-binding protein and transthyretin mRNA levels in visceral yolk sac and liver during fetal development in the rat. Proc. Natl. Acad. Sci. USA. 83, 7330-7334.

Sousa, M.M., Berglund, L. and Saraiva, M.J. 2000. Transthyretin in high density lipoproteins: association with apolipoprotein A-I. J. Lipid Research 41, 58-65.

Sousa, M.M., Vital, D., Ostler, D., Fernandes, R., Pouget-Abadie, J., Carles, D. and Saraiva, M.J. 2000. Apolipoprotein AI and transthyretin as components of amyloid fibrils in a kindred with apoAI Leu178 His amyloidosis. Am. J. Pathol. 156, 1911-1917.

Southwell, B.R., Duan, W., Alcorn, D., Brack, C., Richardson, S.J., Köhrle, J. and Schreiber, G. 1993. Thyroxine transport to the brain: role of protein synthesis by the choroids plexus. Endocrinology 133, 2116-2126.

Sparkes, R.S., Sasaki, H. Mohandas, T., Yoshika, K., Klisak, I., Sakaki, Y., Heinzmann, C. and Simon, M.I. 1987. Assignment of the prealbumin (PALB) gene (familial amyloidotic polyneuropathy) to human chromosome region 18q11.2-q12.1. Hum. Genet. 75, 151-154.

Stabilini, R., Vergani, L., Agostoni, A. and Agostoni, R.P.V. 1968. Influence of age and sex on prealbumin levels. Clin. Chim. Acta 20, 358-359.

Stein, T.D. and Johnson, J.A. 2002. Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. J. Neurosci. 22, 7380-8.

Su, Y., Damjanovsk, S., Shi, Y., and Shi, Y.B. 1999. Molecular and cellular basis of tissue remodeling during amphibian metamorphosis. Histol. Histopathol. 14, 175-183.

Sunde, M., Richardson, S.J., Chang, L. Pettersson, T.M., Schreiber G. and Blake, C.C.F. 1996. The crystal structure of transthyretin from chicken. Eur. J. Biochem. 236, 491-499.

Sundelin, J., Melhus, H., Das, S., Erikson, U., Lind, P. Trägärdh, L., Peterson, P.A. and Rask, L. 1985. The primary structure of rabbit and rat prealbumin and a comparison with the tertiary structure of human prealbumin, J. Biol. Chem. 260, 6481-6487.

Surk, M. I., Schadlow, A.R., Stock, J.M. and Oppenheimer, J.H. 1973. Determination of iodothyronine absorption and conversion of L- thyroxine (T_4) to L-triiodothyronine (T_3) using turnover rate techniques. J. Clin. Invest. 52, 805-811.

Surk, M.I. and Oppenheimer, J.H. 1977. Concentration of L-thyroxine to L-triiodothyronine specifically bound to nuclear receptors in rat liver and kidney. Quantitative evidence favoring a major role of T3 in thyroid hormone action. J. Clin. Invest. 60, 555-562.

Tripathi, G. and Verma, P. 2003. Differential effects of thyroxine on metabolic enzymes and other macromolecules in a freshwater teleost. J. Exp. Zool. 296A, 117-124.

Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A. and Gingeras, T.R. 1987a. Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*. Nucleic Acids Res. 15, 3859-3876.

Tschopp, J.P.F., Sverlow, G., Kosson, R., Craig, W. and Grinna, L. 1987b. High-level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. Bio/Technology 5, 1305-1308.

Tsuzuki, K., Mita, S., Maeda., S., Araki, S. and Shimada, K. 1985. Structure of the human prealbumin gene. J. Biol.Chem. 260, 12224-12227.

Tsuzuki, K., Fukatsu, R., Hayashi, Y., Yoshida, T., Sasaki, N., Takamaru, Y., Yamaguchi, H., Tateno, M., Fuji, N. and Takahata, N. 1997. Amyloid beta protein and transthyretin, sequestrating protein colocalize in normal human kidney. Neurosci.Lett. 222, 163-166.

Tsykin, A. and Schreiber, S. 1993. Sheep thyroxine-binding globulin:cDNA sequence and expression. Mol. Cell Endocrinol. 98, 91-97.

Tu, G.F., Cole, T., Duan, W. and Schreiber, G. 1989. The nucleotide sequence of transthyretin cDNA isolated from sheep choroid plexus cDNA Library. Nucleic Acids Res. 17, 6384.

Twining, S.S. 1984. Fluorescein Iso thiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. 143, 30-43.

Ueno, S., Uemichi, T., Yorifuji, S. and Tarui, S. 1990. A novel variant of transthyretin (Tyr114 to Cys) deduced from the nucleotide sequences of gene fragments from familial amyloidotic polyneuropathy in Japanese sibling casesBiochem. Biophys. Res. Commun. 169, 143-147.

Utiger, R.D. 1995. In: Endocrinology and Metabolism. Felig, P.F., Baxter, J.D. and Frohman, C.A., Eds. McGraw-Hill, New York, 435-519.

Varghese, S. and Oomen, O.V. 1999. Thyroid hormones regulate lipid metabolism in a teleost *Anabas testudineus* (Bloch). Comp. Biochem. Physiol. 124B, 445-450.

Veenhuis, M., van Dijken, J.P. and Harder, W. 1983. The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. Adv. Microb. Physiol. 24, 1-82.

Verma, R., Boleti, E. and George, A.J.T. 1998. Antibody engineering: Comparison of bacterial, yeast, insect and mammalian expression system. J. Immuno. Methods 216, 165-181.

Wagasugi, S., Maeda, S., Shimada, K., Nakashima, H. and Migita, S. 1985. Structural comparisons between mouse and human prealbumin. J. Biochem. 98, 1707-1714.

Warden, B.A. and Geise, R.W. 1984. Soluble antibody affinity chromatography techniques investigated with ultratrace 125I-thyroxine. J. Chromatogr. 314, 295-302.

White, C.E., Hunter, M.J., Meininger, D.P., White, L.R. and Komives, E.A. 1995. Large-scale expression, purification and characterization of small fragments of thrombomodulin: the roles of the sixth domain and of methionine 388. Protein Eng. 8, 1177-1187.

Whitelam, G.C. 1995. The production of recombinant proteins in plants. J. Sci. Food Agric. 68, 1-9.

Wikipedia. Wikipedia-The Free Encyclopedia. Triiodothyronine. 2009.

http://en.wikipedia.org/wiki/Triiodothyronine (accessed 23/01/09), part of Wikipedia. http://en.wikipedia.org (accessed 23/01/09).

Woeber, K.A. and Ingbar, S.H. 1968. The contribution of thyroxine-binding prealbumin to the binding of thyroxine in human serum, as assesses by immunoadsorption. J. Clin. Invest. 47. 1710-1721.

Wood, M.J. and Komives, E.A. 1999. Production of large quantities of isotopically labeled protein in Pichia pastoris by fermentation. J. Biomol. NMR 13, 149-159

Wojtczak, A., Cody, V., Luft, J. R. and Paugborn, W. 1996. Structure of human transthyretin complexed with thyroxine at 2.0 Å resolution and 3′,5′-dinitro-*N*-acetyl-L-thyronine at 2.2 Å resolution. Acta Crystallogr. D52, 758-765.

Wojtczak, A. 1997. Crystal structure of rat transthyretin at 2.5 Å resolution: First report on a unique tetrameric structure. Acta Biochim. Pol. 44, 505-518.

Xiao, S., Pollock, H.G., Taurog, A. and Rawitch, A.B. 1995. Characterization of hormonogenic sites in an N-terminal, cyanogens bromide fragment of human thyroglobulin. Arch. Biochem. Biophys. 327, 61-70.

Yamaguchi, H., Sugihara, S., Ishiguro, S., Takashima, A. and Hirai, S. 1995. Immunohistochemical analysis of COOH-termini of amyloid beta protein(Aβ) using end-specific antisera for Aβ40 and Aβ42 in Alzheimer's disease and normal aging. Amyloid Int. J. Exp. Clin. Invest. 2, 7-16.

Yamano, K., Miwa, S., Obinata, T. and Inui, Y. 1991. Thyroid hormone regulates developmental changes in muscle during flounder metamorphosis. Gen. Comp. Endocrinol. 81, 464-472.

Yamauchi, K., Kasahara, T., Hayashi, H. and Horiuchi, R. 1993. Purification and characterization of a 3, 5, 3′,-L-triiodothyronine-specific binding protein from

bullfrog tadpole plasma: a homolog of mamamalian transthyretin. Endocrinology 132, 2254-2261.

Yamauchi, K., Takeuchi, H., Overall, M., Dziadek, M., Munro, S.L. and Schreiber, G. 1998. Structure characteristics of bullfrog (*Rana catesbeiana*) transthyretin and its cDNA: Comparison of its pattern of expression during metamorphosis with that of lipocalin. Eur. J. Biochem. 256, 287-296.

Yamauchi, K., Nakajima, J., Hayashi, H. and Hara, A. 1999. Purification and characterization of thyroid-hormone-binding protein from masu salmon serum:A homolog of higher vertebrate transthyretin. Eur. J. Biochem. 265, 944-949.

Yen, P.M. 2001. Physiological and molecular basis of thyroid hormone action. Physiol. Rev. 81, 1097-1142.

Zanotti, G., Ottonello, S., Berni, R. and Monaco, H.L. 1993. Crystal structure of the trigonal form of human plasma retinol-binding protein at 2.5 Å resolution. J. Mol. Biol. 230, 613-624.

Zhang, J. and Lazar, M.A. 2000. The mechanism of action of thyroid hormones. Annu. Rev. Physiol. 62, 439-66.

Zheng, W., Deane, R., Redzic, Z., Preston, J.E. and Segal, M.B. 2003. Transport of L-[^{125}I]thyroxine by in situ perfused ovine choroids plexus: Inhibition by lead exposure. J. Toxicol. Environ. Health 66, 435-451.

VITAE

Name Miss Ladda Leelawatwattana

Student ID 4723013

Education Attainment

Degree	Name of Institution	Year of Graduation
B.Sc.(Aquatic Science)	Prince of Songkla University	1999
M.Sc.(Biochemistry)	Prince of Songkla University	2003

Scholarship Awards during Enrolment

University Academic Excellence Strengthening Program in Biochemistry, Prince of Songkla University.

List of Publication and Proceedings

Prapunpoj, P., Leelawatwattana L., Schreiber, G and Richardson, S.J. 2006. Change in structure of the N-terminal region of transthyretin produces change in affinity of transthyretin to T_4 and T_3. FEBS J. 273, 4013-4023.

Leelawatwattana L. and Prapunpoj, P. 2007. Synthesis in *Pichia pastoris* and Proteolytic Property of Recombinant Chimeric Transthyretins. The 7th National Graduate Research Conference Grad-Research 2007. Prince of Songkla University, Surathani Campus, Thailand, May 4-5, 2007, 122.

Leelawatwattana L. and Prapunpoj, P 2008. Synthesis and Characterization of Chimeric Transthyretins. The 9th National Grad Research Conference at Burapha University, March 14 -15, 2008.