# CHAPTER 1

# INTRODUCTION

## Introduction

Transthyretin (TTR), previously referred to as prealbumin, is one of the three major thyroid hormone binding proteins found in plasma of larger mammals. It was found in both serum and cerebrospinal fluid (CSF) of human (Kabat *et al.,* 1942a, b) as a tetramer of four identical β-sheet rich subunits of which were composed 127 amino acid residues (Blake *et al*., 1974, 1978). Since the interactions between monomers predominantly involve hydrogen bonding between the β-strands, this high content of the β-structure contributes to an extraordinary stability of the TTR subunit (Branch *et al.,* 1971, 1972). The gene encoding for the TTR subunit has been localized on chromosome 18 (Wallace et al., 1985). It was found spanned approximately 7.0 kilobase (kb) and comprised four exons and three introns (Sasaki *et al*., 1985) in which each exon contains approximately 200 base pairs (bp) in length. Recently, TTR has been demonstrated as the most abundant protein component of amyloid fibrils (Costa *et al*., 1978) that lead to a group of diseases called amyloidosis. Typical manifestations of the disease caused by deposition of the TTR amyloids are peripheral neuropathy, cardiomyopathy, carpal tunnel syndrome, and vitreous opacities. Up to date, over eighty genetic TTR variants derived from a single amino acid substitutions have been reported (for review, sees Schwarzman *et al*., 2004). Most of them were observed in patients with two related clinical syndromes, familial amyloidotic polyneuropathy (FAP), which is found TTR amyloids deposition systemically in the peripheral nervous system, and familial amyloidosis cardiomyopathy (FAC). In addition, TTR with an entirely normal amino acid sequence was found in patients with senile systemic amyloidosis (Westermark *et al*., 1990).

FAP and FAC were described as the autosomal dominant inherited diseases caused by different single point mutations of the TTR gene. However, role of TTR mutations in the amyloidogenesis is not well understood and different mutations are often associated with distinct clinical symptoms. Several reports suggested that point mutations of TTR gene could change the conformation of the protein to an intermediate oligomer with an extensive β-sheet. Up to now, over 80 different mutations in TTR have been reported relating FAP. Without a proper therapy, TTR amyloidoses can result in the fatal course, leading to death within 10 to 15 years. Orthopic liver transplantation is the most powerful therapy available nowadays. The treatment was shown to virtually eliminate TTR variant from plasma of the patients with FAP. However, it does not provide a practical means of treatment for a large number of patients. The cardiac amyloidosis could progress and its effect on autonomic dysfunction has not been established (Adams *et al*., 2000). Thus, other therapeutic strategies, e.g. a specific blocking an expression of the mutant TTR gene and stabilization of native conformation of the TTR with chemicals such as 4′-deoxy-4′-iodoxorubicin (IDOX), tetracycline and nitrophenols (Cardoso *et al*., 2003) are being pursued.

Frequency and clinical manifestations including age of onset, sex ratio and duration of the disease generated by deposition of the TTR amyloids are varied among populations. For examples, among the pathogenic TTR identified so far, Val30Met (V30M) is the most frequent variant (Saraiva, 1984, 2001), vastly found in Portuguese and Swedish. In contrast, heterogeneity of the point mutations of TTR that result in more than 20 different amino acid substitutions including Val30Met has been reported among Japanese (Andersson, 1970; Araki, 1984; Ikeda *et al.,* 2002). Similar to that observed in French (Planté-Bordeneuve *et al.,* 1998), heterogeneity incident of Leu55Pro and Val122Ile on FAC was reported in many other populations including Taiwanese and African Americans (Jacobson *et al*., 1992; Yamamoto *et al*., 1994), putting these people to a significant risk for congestive heart failure. In Thailand, no genetic variation of the TTR gene has been studied so far. To accomplish for direct diagnosis and specific treatment for Thai patients, information on type and characteristics of TTR variant distribute in the area is essential.

## Review of Literatures

### 1. Transthyretin (TTR)

#### 1.1. General

TTR is one of the three major thyroid hormone-binding proteins found in plasma of larger mammals. It was first found in both serum and cerebrospinal fluid (CSF) (Kabat *et al.,* 1942 a, b) of human. TTR was formerly referred to as thyroxine-binding prealbumin. It is the only protein in human plasma found to migrate ahead of albumin during electrophoresis of the whole plasma in barbiturate buffer at pH 8.6 (Ingbar, 1958; Pages *et al.,* 1973). Name of TTR became more accepted when the protein was shown to be a carrier of thyroxine (Robbins *et al*., 1957; Oppenheimer, 1968). Electrophoretic mobility and thyroxine-binding capacity of TTR are distinct from the other thyroid hormone-binding proteins (Ingbar, 1958).

TTR is widely distributed among vertebrate species. It is the major thyroid hormone-binding protein found in blood of all adult bird, herbivorous marsupials and small eutherians, but it was not found in plasma of adult Australian polyprotodont marsupials, reptiles and adult amphibians (for review see Schreiber and Richardson, 1997; Schreiber *et al*., 1999). It is also the major thyroid hormone-binding protein in the CSF of reptiles, birds and mammals (Harms *et al.*, 1991; Richardson *et al*., 1994), and may have a role in distribution of T4 and T3 to and within the central nervous system (Dickson *et al*., 1987a; Schreiber *et al*., 1990; Southwell *et al*., 1993).

#### 1.2. Structure and chemical properties of TTR

TTR is a globular plasma protein comprising four identical subunits (Blake *et al*., 1978). It has a molecular mass of ~55 kDa and a subunit mass of ~14 kDa. In human, the TTR subunit consists of 127 amino acid residues (Figure 1.1) (Kanda *et al*., 1974). Most of these amino acid residues are arranged into eight stranded β-sheets, and only 5% of them were found in a short α-helix portion (Figure 1.2 A) (Blake *et al*., 1974). TTR and albumin differ from thyroxine-binding globulin (TBG) in that they are not glycosylated. Heterogeneity of TTR observed in several

animal species (Miller, 1963; Baker *et al*., 1966; Muto and Goodman, 1972; Taylor *et al*., 1975; Bhat *et al*., 1997; Harm *et al*., 1991) was suggested as resulted from other modifications including dihydroxylation, phosphorylation, cysteine-glycine conjugation and glutathionylation (Terazaki *et al.*, 1998). Moreover, heterogeneity of TTR resulted from complex formation of the protein with retinol binding protein (RBP) was also observed (Glover, 1973; Kopelman *et al*., 1976).

Figure 1.1 Amino acid sequence of human TTR

**$NH_2$**-Gly-Pro-Thr-Gly-Thr-Gly-Glu-Ser-Lys-Cys-Pro-Leu-Met-Val-Lys-Val-Leu-Asp-Ala-Val-Arg-Gly-Ser-Pro-Ala-Ile-Asn-Val-Ala-Val-His-Val-Phe-Arg-Lys-Ala-Ala-Asp-Asp-Thr-Trp-Glu-Pro-Phe-Ala-Ser-Gly-Lys-Thr-Ser-Glu-Ser-Gly-Glu-Leu-His-Gly-Leu-Thr-Thr-Glx-Glx-Gln-Phe-Val-Glu-Gly-Ile-Tyr-Lys-Val-Glu-Ile-Asp-Thr-Lys-Ser-Tyr-Trp-Lys-Ala-Leu-Gly-Ile-Ser-Pro-Phe-His-Glu-His-Ala-Glu-Val-Val-Phe-Thr-Ala-Asn-Asp-Ser-Gly-Pro-Arg-Arg-Tyr-Thr-Ile-Ala-Ala-Leu-Leu-Ser-Pro-Tyr-Ser-Tyr-Ser-Thr-Thr-Ala-Val-Val-Thr-Asn-Pro-Lys-Glu-**COOH**

The three-dimentional structure of human plasma TTR has been determined by X-ray diffraction at both 2.5 Å (Blake and Oatley, 1977; Wojtczak, 1997) and 1.8 Å resolutions (Blake *et al*., 1978). It showed that about 45% of the amino acid residues of each TTR monomer are organized into eight β-sheets, which form into two four-strand β-sheets that denoted as DAGH and CBEF. These eight strands of the β-sheet, then, form a β sandwich structure connected by the A-B loops (Blake *et al*., 1978). A short α-helix was found located at the end of strand E (Figure 1.2 B). The rest of amino acid residues were found located in seven loops that connect the eight β-strands, and segments of N-terminus (10 amino acid residues) and C-terminus (5 amino acid residues) (Hamilton *et a*l., 1993), which located outside the core structure of the tetramer. Two monomers of TTR joined edge-to-edge to form a dimer resulted in a pair of twisted eight-stranded of the β-sheets, an inner

(DAGHH′G′A′D′) and an outer (CBEFF′E′B′C). The interactions between monomers involve hydrogen bonding between two F strands (F, F′) and two H strands (H, H′), however, H-strands are more extensively hydrogen bonded than the F strands. The tetrameric structure of TTR is formed from two dimers via hydrophobic interactions between amino acid residues on the A-B loop and the H strand of the opposite dimer (Figure 1.3). High content of β-structure (Branch *et al*., 1971, 1972), interactions between monomers to form dimers and those between dimers to form tetramer contribute to make TTR an extraordinary stability. In comparison, TTR in the tetrameric form is less stable than the dimer (Blake *et al*., 1978).

A large central channel with approximately to 10 Å in diameter and 50 Å long and with sterically equivalent in thyroid hormone binding, but with differ in their relative binding affinities, is formed as a consequence of the tetrahedral arrangement of the TTR subunits (between DAGH) (Blake *et al*., 1978). Amino acid residues that involve in ligand binding were located in strand A (residue 14 to 18), D (residue 53 to 56) and G and H (residue 105 to 122) (Neumann *et al*., 2001). The chemical nature of the channel consists of three main elements. First, a hydrophilic center that forming from the hydroxyl groups of Ser and Thr residues, and associated bound-water molecules. Second, a hydrophobic patch that formed by the methyl group of Leu, Thr, Ala, Val and a group of charged residue such as Lys, Glu and His at which constitute around the entrance of the channel (for review see Blake, 1981; Klabunde *et al*., 2000). And, finally, a negative cooperate binding of two molecules thyroid hormones with the amino acid residues inside the channel (Neumann *et al*., 2001; Blake *et al*., 1974). It revealed that only one binding site of TTR is occupied by thyroid hormone under physiological condition (Page *et al*., 1973; Nilsson *et al.*, 1975).

The tetrameric form of TTR is stable and non-dissociated even in strong acidic or 0.1% sodium dodecyl sulfate (SDS) solution. But at some conditions such as at low pH (3.5 to 5.0) or high molar of denaturing agent, e.g. 4 M to 6 M guanidine hydrochloride (Guanidine-HCl), TTR tetramer were slowly dissociated into monomers (Kelly *et al*., 1997; Lai *et al*., 1997). Dissociation into dimers and incomplete dissociation into monomers of TTR tetramer were also reported can occur in a solution of 5% SDS without heating and in 8 M urea, respectively (Rask *et al*.,

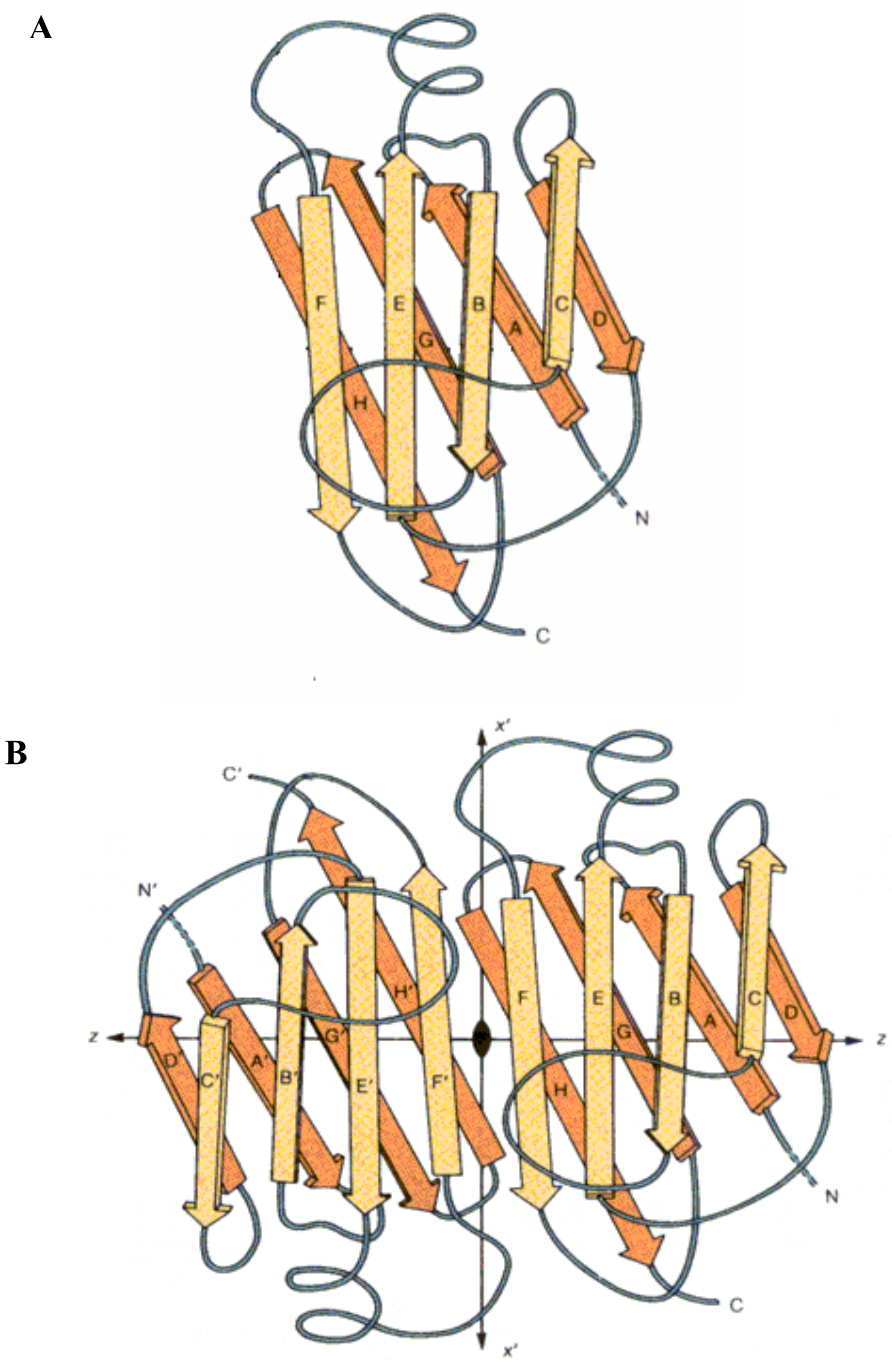


**Figure 1.2 Structure of human TTR monomer (A) and dimer (B).**
Each TTR monomer comprises two β-sheets, which are formed from DAGH and CBEF strands, and one α-helix. Whereas, face-to-face interaction of the two β-sheets through a hydrogen bonding between HH' and FF' strands is the key for formation of dimer (Blake *et al.*, 1978).

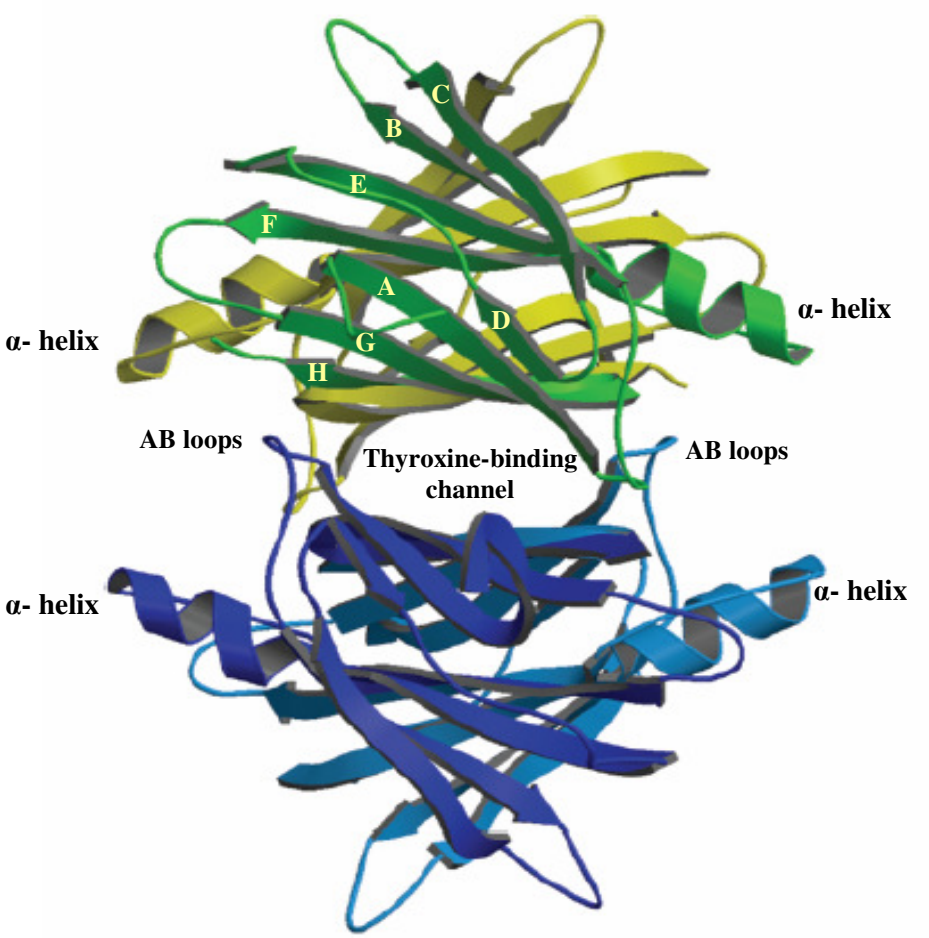


**Figure 1.3 The ribbon model of TTR tetramer**

Two dimers of TTR subunits link together via AB loops in forming a tetramer and the thyroxin-binding channel was generated from the linking of the dimers (From Monaco, 2002)

1971). In addition, the tetrameric dissociation into monomer of TTR was also suggested to occur at pH 7.0 and at nearly physiological ionic strength upon dilution in the sub molecular range (Quintas *et al*., 1997; 1999). TTR lost the thyroxine binding activity when treated with 6 M urea, however, its binding activity could be restored when urea was removed from the protein (Raz and Goodmann, 1969).

### 1.3. Gene structure of human TTR

Each human TTR monomer is encoded by a single-copy gene (Sparkes e*t al*., 1987) to located on the long arm of chromosome 18 (Wallace *et al*., 1985). It was found span to about 6.9 kilobases and to contain four exons and three introns (Figure 1.4) (Sasaki *et al*., 1985). The nucleotide sequences at 5′ and 3′ flanking exon/intron border regions and introns of the TTR gene have been analyzed (Sasaki *et al*., 1985; Fung *et al*., 1988; Motojima and Goto., 1990). It showed that nucleotide sequence of the TTR exon 1 was 95 base pairs (bp), which encodes twenty amino acid residues of a signal peptide and three amino acid residues of the mature protein. Whereas, exon 2, exon 3 and exon 4 contain 131, 136 and 254 bp that encode for 44, 45 and 35 amino acid residues, respectively. The distance between the transcriptional initiate site and the poly (A) site is 6,931 bp. The sequence of poly (A) tail is located 123 bp downstream of the coding region (Sasaki *et al*., 1985). Lengths of the three introns are 934, 2090 and 3308 bp, respectively. The consensus sequences for splicing, GT and AG, were found at the boundaries of all three introns (Breathnatch *et al*., 1978). Two sequence copies of *Alu* family were found in intron 2 and 3 of the TTR gene, and it was suggested to involve in the gene expression or regulation. The repeated sequences at 3′ untranslated region were found almost identical to the core sequences. However, biological function of these repeated sequences is still unknown. The sequence of TTR gene is highly conserved through evolution. More than 80% identity in the sequence was reported among the mammalian TTRs (Wakasugi *et al*., 1985; Schreiber and Richardson, 1997).

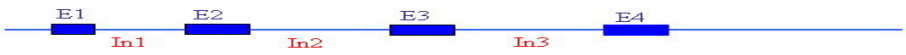


**Figure 1.4 Structural diagram of TTR gene**

Close boxes with E1, E2, E3 and E4 on top represent TTR exon 1, 2, 3 and 4, respectively. Blue line with In1, In2 and In3 underneath represent TTR intron1, 2 and 3, respectively.

### 1.4. Synthesis and distribution of TTR

TTR is mainly synthesized in liver (Felding and Fex, 1982) and epithelial cells of the choroid plexus (Aleshire *et al.,* 1983). In addition, it is also found synthesized in other cells including the retinol pigment epithelial cells (Martone *et al.*, 1988; Herbert *et al.,* 1991; Ong *et al.,* 1994). TTR in blood circulation is mainly produced in hepatocytes, and then secreted into the blood (Dickson *et al.,* 1986). It is synthesized as a pre-protein with a signal peptide at N-terminus, which is cleaved during translocation of the protein into an endoplasmic reticulum (ER), and within the ER of hepatocytes, TTR tetramer is formed (Bellovino *et al.,* 1996; 1998). TTR is distributed throughout the body and in equilibrium with the interstitial fluid by penetrating the capillaries. The plasma level of TTR increases successively after birth and reaches the adult level, i.e. 250 µg/ml (Smith and Goodman, 1971). However, the concentration of plasma TTR decreases after the fifth decade of life.

TTR in liver is one of a negative acute phase reactant. The rate of TTR synthesis decreases during acute phase response to trauma and chronic inflammation (Birch and Schreiber, 1986; Milland *et al.,* 1990; Richardsos *et al.,* 1998). Low concentration of TTR may result in higher concentration of free ligand, e.g. thyroxine and retinol, which lead to malnutrition. In addition, it can reflect the metabolic competence of body (for review see Raghu and Sivakumar, 2004). Although, synthesis of TTR by liver may be altered under stress condition, the synthesis of TTR in choroid plexus was very slightly affected (Dickson *et al.,* 1982). This indicated that the synthesis of TTR in liver and choroid plexus is independently regulated during the acute phase response (Dickson *et al*., 1986).

Choroid plexus, an epithelial membrane component of blood-brain barrier, is the main synthesis site of TTR found in CSF (Cserr, 1971). All of TTR synthesis by the choroid plexus is secreted towards the brain and has been proposed to be involved in transport of thyroid hormone, T4 but not T3, from blood to the brain (Dickson *et al.*, 1987). Localization of TTR in choroid plexus was first demonstrated using an immunohistochemical technique (Aleshine *et al.,* 1983), and it was found distributed in cytoplasm and epithelial cell of the choroid plexus (Herbert *et al.,* 1986). TTR mRNA was located in the epithelial cells prior to being secreted into the CSF (Southwell *et al.,* 1993). Its concentration in choroid plexus was much higher

than that in liver (Schreiber *et al.,* 1990). About 50% of total secreted protein and about 12% of the synthesized protein in choroid plexus is TTR (Dickson *et al.,* 1986). In mammal and avian, the TTR gene was expressed in the cells of the choroid plexus in early stage of embryogenesis before the formation of blood brain-barrier (Southwell *et al.,* 1991) and during fetal development (Thomas *et al.,* 1988). A correlation between growth rate of the brain and expression level of TTR gene was evidence in growing animal (Southwell *et al.,* 1991).

### 1.5. Functions of TTR

The most well-known physiological function of TTR is a protein transporter for thyroid hormones and vitamin A. Thyroid hormones, both T4 and T3, directly bind to TTR whereas vitamin A (in retinol form) is bound to TTR through the mediation of retinol-binding protein (RBP).

#### 1.5.1. As a thyroid hormone distributor protein

TTR is the one of thyroid hormones binding protein in plasma of higher vertebrates, besides thyroid hormone binding globulin (TBG) and albumin. In human, binding affinity of TTR to thyroid hormones, in particular T4, is less than TBG but higher than albumin (for review sees Schreiber and Richardson, 1997). About 10-15% (Palha, 2002) and up to 80% (Hagen and Elliot, 1973) of T4 in human plasma and in central nervous system, respectively, are bound and transported by TTR. Human TTR has higher affinity for T4 than T3, i.e. T4 bound to TTR about 10 times higher than T3. However, this binding affinity was change during evolution of vertebrates (for review see Schreiber and Richardson, 1997; Schreiber *et al.,* 1999)

TTR has two binding sites for thyroid hormones (Blake *et al.,* 1978). At the physiological condition, only one site is occupied by the hormone molecule due to the negative cooperativity of thyroid hormone binding. By equilibrium dialysis measurement, the association constant ($K_a$) was $1.0x10^8$ $M^{-1}$ and $9.5x10^5$ $M^{-1}$ for the first and second ligand binding sites, respectively (Ferguson *et al.,* 1975), suggesting that binding of the first ligand was much stronger than the second ligand. This cooperative effect is involved in the conformational changes induced by ligand binding to TTR molecule (Neumann *et al.,* 2001). By comparing the TTR

channel diameter of the two binding sites, it was reveal that binding of the hormone to the first site can changed diameter of the second. The outer and inner pockets of the channel (strand A, D and H) were wider while the middle part (strand G) was collapsed (Neumann *et al.,* 2001). Therefore, the second hormone molecule bound to the binding site with less strength.

The tissue distribution of TTR synthesis was observed by intravenous injection of $^{125}$I-labeled thyroid hormones to various tissues (Dickson *et al.,* 1987). T4 was found first strongly accumulation in the choroid plexus. Then, it was transported across the blood brain barrier into CSF by binding with TTR (Southwell *et al.,* 1993). This revealed the hypothetical mechanisms for T4 transport from blood to brain in such a way that T4 enter to the brain by moving across the epithelial cells of choroid plexus or moving across the cerebral endothelial cells. In moving across, T4 bound to TTR that locates within the choroid plexus or within CSF (Figure 1.5). In addition, as the concentration of TTR in CSF was related to the total concentration of T4 that transported to the brain and between tissue and extracellular fluid, the mechanism of regulation of T4 level in the brain was proposed.

### 1.5.2. As a carrier protein for retinol via binding to retinol-binding protein (RBP)

TTR also binds and transports vitamin A or retinol through the binding of a vitamin A carrier protein named retinol-binding protein (RBP), which is a 21-kDa single polypeptide chain protein found in plasma of vertebrates. The TTR-RBP complex transports retinol from the main storage in liver to target cells in peripheral tissues. It was shown that without binding to TTR, RBP can be eliminated by glomerular filtration in kidney because of its small molecule. Therefore, the binding of RBP to TTR was thought to prevent the loss of RBP and retinol through glomerular filtration in kidney (Kanai *et al*., 1968; Goodman and Raz, 1972). In human plasma, about 40% of TTR circulates as the complex with RBP (Smith and Goodman, 1971), while less than 1% circulates as the complex with T4 (Woeber and Ingbar, 1968). The complex formation of TTR and RBP was found occurred at physiological pH and involved with a hydrophobic interaction (Peterson, 1971). The complex dissociated readily at low ionic strength and in the presence of 6 M urea. .In

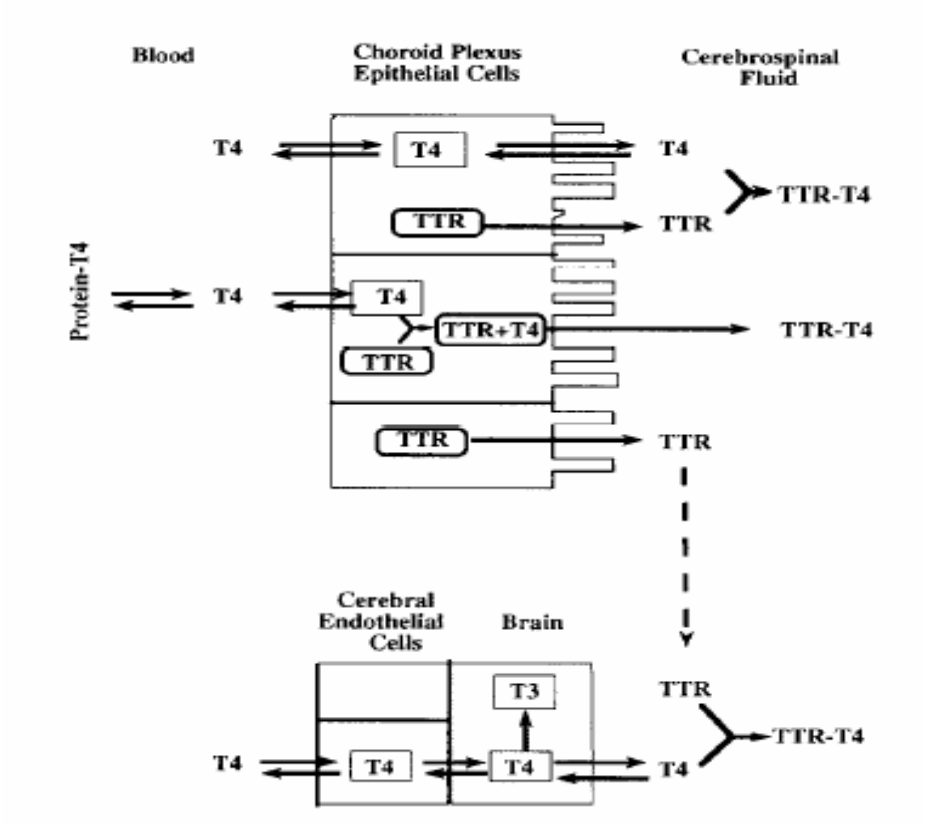


**Figure 1.5** **The hypothetical mechanisms for the role of TTR within choroid plexus in moving T4 from the blood to brain** (from Southwell *et al.,* 1993).

The diagram shows the hypothetical routes for T4 movement from blood to brain and cerebrospinal fluid. T4 may enter the brain by moving across the choroid plexus epithelial cell (the upper) or moving across the cerebral endothelial cells (the lower). T4 does not undergo deiodination in the choroid plexus but conversion of T4 to T3 is occurred within the brain tissue by deiodinases.

human plasma, TTR and RBP formed complex with 1:1 molar ratio even though the *in vitro* studying suggested more than one binding sites for RBP (one to four sites) was on a TTR molecule (Raghu *et al*., 2003). This was probably due to the limit concentration of RBP in comparing to that of TTR. The binding sites for T4 and RBP are independent from each other (Eneqvist *et al*., 2001). The binding of RBP did not change the binding capacity of TTR to T4 (Nilsson and Peterson, 1971).

### 1.5.3. Other functions

A thymic hormone-like activity influence of TTR was demonstrated in spleen cells of thymectomized rats (Burton *et al.,* 1978, 1985). It indicated that TTR possessed immunopotentiating properties *in vitro* and *in vivo* (Burton *et al.,* 1985). Induction with TTR enhanced synthesis of IgM, and a decrease in the specific activity of thymocyte terminal deoxynucleotidyl transferase was also observed. Moreover, the amino-terminal domain of the TTR molecule was shown containing a property similar to T-lymphocytes (Burton *et al.,* 1987).

TTR has been shown interacting with perlecan, an important constituent of basement membranes and connective tissues (Iozzo *et al.,* 1994) and has been identified as a common proteoglycan componebt occurring in several types of amyloidosis (Kisilevsky *et al.,* 1992; Magnus *et al.,* 1992). It has been proposed that the binding of perlecan to TTR depends on hydrophobic interactions between the two proteins. Binding of perlecan to TTR resulted in sequestering the protein from the retinol-RBP-TTR complex (Smeland *et al.,* 1997), thus affecting metabolism of retinol. Moreover, interaction between TTR and perlecan was suggested favor with the co-localization of these two proteins in the TTR-associated amyloidotic deposits (Smeland *et al.,* 1997).

Recently, TTR has been reported having the proteolytic activity (Liz et al., 2004). It was shown can proteolytical process the C-terminal of apolipoprotein A-1 and was suggested may interact with other substrates *in vivo* under physiological and pathological condition.

## 2. Amyloidosis

Amylodosis is a group of diseases in which proteins or protein fragments change from their native soluble forms into an insoluble fibril called "amyloid". The amyloid fibrils have been found accumulated in a variety of organs and tissues of the body including peripheral nerves, kidney, gastrointestinal tract, heart and thyroid (Kelly, 1996; Rochet and Lansbury, 2000). The accumulation of large amounts of amyloid fibrils can result in damaging of function and structure of organs. Up to date, 24 different proteins have been identified as the amyloidogenic in human, and they are associated with several disorders including Alzheimer's disease (Selkoe, 1996), Parkinson's disease (Conway *et al*., 1998), familial amyloidotic polyneuropathy (FAP) (Costa *et al*., 1978; Mc Cutchen *et al*., 1995), light chain amyloidosis (Buxbaum and Gallo, 1999), and dialysis-related amyloidosis (Drueke, 2000).

The "amyloid" was first used to describe the cerebral corpora amylacea, a material that was found during histopathological examination of brain (Virchow, 1854), which was later identified as a carbohydrate substance. In 1859, it was shown that protein was a component of amyloid (Friedreich and Kelkulé, 1859). By detection using Congo red, an amorphous proteinaceous aggregates were identified (Bennhold, 1922). The fibrillar ultrastructure of the amyloid fibrils was later revealed by electron microscopy (Cohen and Calkins, 1959).

### 2.1. Nature of amyloid

Amyloid is an insoluble fibrillar protein that extracellularly deposit in tissues or organs. The amyloid fibrils can be formed from several different precursor proteins that share a common ultrastructure. These proteins exhibit as straight and non-branch fibrils with about 7.5 to 10 nm thick but undetermined length, and reveal a superficial twist and two protofibrils (Cohen, 1966). The X-ray diffraction showed that the fibril has a "cross ß" structure in which the constituent strands are arranged perpendicular to the long axis of the fibril (Blake and Serpell, 1996). Moreover, the amyloid fibril showed uniform tintorial properties including apple green birefringence after staining with Congo red (Glenner, 1981; Serpell et al., 1997). The amyloid fibrils showed orange colored fluorescence that is most sensitive (Linke, 2000).

Many mechanisms have been proposed for the conformational change of the precursor protein that leads to the fibril formation. These include point mutation (Saraiva, 2001; Shastry, 2003), overproduction of the precursor protein (Gillmore *et al*., 2001), change in local pH of membrane, oxidation and proteolytic cleavage (Haass and Steiner, 2001). However, several amyloid fibrils have been demonstrated as forming from the wild type of precursor proteins (Westermark *et al.*, 1986). The precursor proteins that adopt a globular folding in their native state require partial unfolding of their tertiary structure prior to the fibril formation. While those unfolded in their native state required a transition to an ordered secondary structure (Rochet and Lansbury, 2000).

### 2.2. Classification of amyloid

The amyloid disease can be divided into two groups according to their distribution in the body, the localized and the systemic amyloidoses. For the localized amyloids, the deposition is restricted to one tissue or organ whereas the systemic amyloids deposit in many different organs or tissues. All amyloid precursor proteins have been identified so far are diverse and unrelated, but produce amyloid deposition with beta-fibrillar as a common structure. The specific precursor proteins and their classification are listed in Table 1.1

### 2.3. TTR amyloidosis

TTR is one of the precursor proteins known to form amyloid fibril (Damas and Saraiva, 2000), which is associated with systemic amyloidosis. The TTR amyloidosis including familial amyloidotic polyneuropathy (FAP) and cardiomyopathy (FAC) are caused by mutation of the TTR gene. Up to date, more than 80 point mutations of TTR have been identified and most of them were revealed associated with FAP. Only about 12 of the mutations are nonpathogenic (Connors *et al.,* 2003; Saraiva, 2001). TTR mutations have been reported to be a non-causative molecule for the senile systemic amyloidosis (SSA). However, this disease is a sporadic disorder resulted from deposition of the wild-type TTR fibril in cardiac and other tissues.

Table 1.1 Human amyloid fibril proteins and their precursors (Westermark *et al*., 2002)

| Amyloid protein | Precursor | Systemic (S) or Localized (L) | Syndrome or tissue of deposition |
|---|---|---|---|
| | Immunoglobulin light | | Primary, Myeloma associated |
| AL | chain | S, L | Primary, Myeloma associated |
| AH | Immunoglobulin heavy | | Familial, Senile systemic |
| | chain | S, L | Hemodialysis |
| ATTR | Transthyretin | S | Secondary, reactive |
| Aß$_2$M | $\beta_2$-microglobulin | S | Familial |
| AA | (Apo)serum AA | S | Familial |
| AApoA-I | Apolipoprotein A-I | S | Familial |
| AApoA-II | Apolipoprotein A-II | S | Familial |
| AGel | Gelsolin | S | Familial |
| ALys | Lysozyme | S | Familial |
| AFib | Fibrinogen α-chain | S | Familial |
| ACys | Cystatin C | S | Familial |
| ABri | ABriPP | L | Familial dementia |
| ADan | ADanPP | L | Familial dementia |
| Aß | Aβ protein precursor (AβPP) | L | Alzheimer's disease, aging |
| APrP | Prion protein | L | Spongioform encephalopathies |
| ACal | (Pro)calcitonin | L | C-cell thyroid tumors |
| AIAPP | Islet amyloid polypeptide | L | Islets of Langerhans insulinomas |
| AANF | Atrial natriuretic factor | L | Cardiac atrial, |
| APro | Prolactin | L | Aging pituitary, Prolactinomas |
| AIns | Insulin | L | Iatrogenic |
| AMed | Lactedherin | L | Senile aortic, media |
| Aker | Kereto-epithelin | L | Cornea; Familial |
| A(tbn) | To be named | L | Pindborg tumors |
| ALac | Lactoferrin | L | Cornea; Familial |

### 2.3.1. Familial amyloidotic polyneuropathy (FAP)

Familial amyloidotic polyneuropathy (FAP) is the most common hereditary systemic amyloidosis. It is identified as an autosomal dominant disorder with peripheral sensorimotor and autonomic neuropathy (Andrade, 1952). It is well known that amyloidogenic TTR, which is resulted from the point mutations, is a major constituent of the amyloid that deposits in tissue of the FAP patients. The disorder was first observed in the Portuguese (Andrade, 1952). The amyloid fibrils in FAP were shown extracellularly systemic deposited throughout the connective tissue with the exception of brain and liver parenchyma. It affected particularly the peripheral nervous system (Coimbra and Andrade, 1971a, b) and led to dysfunction of organs and ultimately death. The symptom onset of FAP usually occurs in the third to the forth decade of life. The symptom usually begins with a neuropathy of the sensory peripheral in lower limbs in which pain and temperature sensation are most severely effected. The following symptom is the motor impairments, which leads to wasting and weakness (Andrade, 1952; Booth *et al*., 1998; Misrahi *et al*., 1998). Most of FAP patients involve with an early and severe autonomic nervous system, commonly manifested by dyshydrosis, sexual impotence, alternating diarrhea and constipation as well as orthostatic hypotension and urinary bladder dysfunction (Canijo and Andrade, 1969; Guimarães *et al*., 1980; Alves *et al*., 1997b; Ando and Suhr, 1998).

The biochemical nature of amyloid deposits was elucidated. It showed mutated TTR as the main protein constituent of the amyloid deposits in FAP (Costa *et al*., 1978). Among the mutations, TTR Val30Met, whose valine at position 30 was replaced by methionine, is the most common. Later, Val30Met has been found throughout the world, however, in endemic areas. It is frequently found in northern of Sweden (Andersson, 1976; Holmgren *et al*., 1994), northern of Portugal (Andrade, 1952; Alves *et al*., 1997a), and Japan (Araki *et al*., 1968; Araki, 1984). Prevalence and age of the disease onset vary among and within populations. In Portugal, prevalence of Val30Met is high, i.e. at least 80%, and the symptoms typically develop before the age of 40 years (Sousa *et al*., 1995). A slightly lower prevalence, to approximate 5%, and later onset, usually after the age of 50 years, of the disease were reported in Swedish carriers (Andersson, 1976; Sousa *et al*., 1993). Onset of the disease reported in the Japanese was either early or late (Tashima *et al*., 1995).

Incidentally, the Val30Met homozygous did not show more severe form of FAP or some did not, even, develop FAP (Holmgren *et al*., 1988; Yoshinaga *et al*., 1992). This indicates that mutation itself is necessary but not sufficient to cause FAP. Other unidentified genetic or environmental factors might contribute to the pathogenesis of the disease.

Up to date, more than 80 mutations of TTR gene have been reported in association with human amyloidosis (see Table 1.2) (Saraiva, 2001), and most of them are connected to FAP with an indistinguishable clinical symptom from the original description of the disease (Toyooka *et al*., 1995; Booth *et al*., 1998; Misrahi *et al*., 1998; de Carvalho *et al*., 2000). Whereas, the others may contribute to a variety of phenotype including simultaneous neuropathy and cardiomyopathy, carpal tunnel syndrome, predominance of vitreous TTR deposition, and involvement of leptomeningeal. Only a few TTR mutations are related to cardiomyopathy without neurological symptoms. The most common TTR mutation associated with cardiac amyloidosis (FAC) is Val122Ile, frequently found in the African-Americans. The allele frequency of Val122Ile among the population was about 12% (Jacobson *et al*., 1997)

Table 1.2. TTR amyloidogenic variants (from Saraiva., 2001)

| Mutation | Codon change | | Predominant clinical feature | Origin |
|---|---|---|---|---|
| Cys10Arg | TGT | CGT | PN, AN, Eye | Hungary |
| Leu 12Pro | CTG | CCG | LM, PN, AN | UK |
| Asp18Glu | GAT | GAG | PN, AN | Columbia |
| Asp18Gly | GAT | GGT | LM | Hungary |
| Val20Ile | GTC | ATC | Heart | Germany |
| Ser23Asn | AGT | AAT | Heart | Portugal |
| Pro24Ser | CCT | TCT | Heart, CTS, PN | USA |
| Val28Met | GTG | ATG | PN, AN | Portugal |
| Val30Met | GTG | ATG | PN, AN, Eye | Several |
| Val30Ala | GTG | GCG | Heart, AN | Germany |
| Val30Leu | GTG | CTG | PN, AN | Japan |
| Val30Gly | GTG | GGG | LM, Eye | France |
| Phe33Ile | TTC | ATC | PN, Eye | Poland |
| Phe33Leu | TTC | CTC | PN, AN | Poland |
| Phe33Val | TTC | GTC | PN, AN | UK |
| Arg34Thr | AGA | ACA | PN, Heart | Italy |
| Lys35Asn | AAG | AAC | PN, AN, Heart | France |
| Ala36Pro | GCT | CCT | PN, Eye | Greece |
| Asp38Ala | GAT | GCT | PN, Heart | Japan |
| Glu42Gly | GAG | GGG | PN, AN | Japan |
| Glu42Asp | GAG | GAT | Heart | France |
| Phe44Ser | TTT | TCT | PN, AN, Heart | Ireland |
| Ala 45Asp | GCC | GAC | Heart | Italy |
| Ala45Ser | GCC | UCC | Heart | Sweden |
| Ala45Thr | GCC | ACC | Heart | Italy |
| Gly47Arg | GGG | CGG | PN, AN | Japan |
| Gly47Ala | GGG | GCG | Heart, PN, AN | Italy |

Table 1.2 . (continued)

| Mutation | Codon change | | Predominant clinical feature | Origin |
|---|---|---|---|---|
| Gly47Glu | GGG | GAG | PN | Germany |
| Thr49Ala | ACC | GCC | Heart, PN | Italy |
| Thr49Ile | ACC | ATC | PN, Heart | Japan |
| Ser50Arg | AGT | AGG | PN, AN | Japan |
| Ser50Ile | AGT | ATT | Heart, PN, AN | Japan |
| Glu51Gly | GAG | GGG | Heart | USA |
| Ser52Pro | TCT | CCT | PN, AN, Heart | UK |
| Gly53Glu | GGA | GAA | LM, Heart | France |
| Glu54Gly | GAG | GGG | PN, AN | UK |
| Glu54Lys | GAG | GAA | PN, AN, Heart | Japan |
| Leu55Arg | CTG | CGG | LM, PN | Germany |
| Leu55Pro | CTG | CCG | PN, Heart, AN | Taiwan |
| His56Arg | CAT | CGT | Heart | USA |
| Leu58His | CTC | CAC | CTS, Heart | Germany |
| Leu58Arg | CTC | CGC | CTS, AN, Eye | Japan |
| Thr59Lys | ACA | AAA | Heart, PN | Italy |
| Thr60Ala | ACT | GCT | Heart, CTS | Ireland |
| Glu61Lys | GAG | AAG | PN | Japan |
| Phe64Leu | TTT | CTT | PN, CTS, Heart | Italy |
| Phe64Ser | TTT | TCT | LM, PN, Eye | Italy |
| Ile68Leu | ATA | TTA | Heart | Germany |
| Tyr69His | TAC | CAC | Eye | Scotland |
| Lys70Asn | AAA | AAC | CTS, PN, Eye | Germany |
| Val71Ala | GTG | GCG | PN, Eye | Spain |
| Ile73Val | ATA | GTA | PN, AN | Bangladesh |
| Ser77Phe | TCT | TTT | PN | France |
| Ser77Tyr | TCT | TAT | PN | Germany |

Table 1.2. (continued)

| Mutation | Codon change | | Predominant clinical feature | Origin |
|---|---|---|---|---|
| Ile84Asn | ATC | AAC | Eye, Heart | Italy |
| Ile84Thr | ATC | ACC | Heart, PN, AN | Germany |
| Glu89Gln | GAG | CAG | PN, Heart | Italy |
| Gku89Lys | GAG | AAG | PN, Heart | USA |
| Ala91Ser | GCA | TCA | PN, CTS, Heart | France |
| Ala97Gly | GCC | GGC | Heart, PN | Japan |
| Ala97Ser | GCC | TCC | PN, Heart | France |
| Ile107Val | ATT | GTT | Heart, CTS, PN | Germany |
| Ile107Met | ATT | ATG | PN, Heart | Germany |
| Ala109Ser | GCC | TCC | PN | Japan |
| Leu111Met | CTG | ATG | Heart | Denmark |
| Ser112Ile | AGC | ATC | PN, Heart | Italy |
| Tyr114Cys | TAC | TGC | PN, AN, Eye | Japan |
| Tyr114His | TAC | CAC | CTS | Japan |
| Tyr116Ser | TAT | TCT | PN, CTS | France |
| Ala120Ser | GCT | TCT | Heart, PN, AN | Africa |
| Val122Ile | GTC | ATC | Heart | Africa |
| Val122del | GTC | Loss | Heart, PN, CTS | Equador/Spain |
| Val122Ala | GTC | GCC | Heart, Eye, PN | UK |

AN, autonomic neuropathy; CTS, carpal tunnel syndrome; Eye, vitreous deposition; PN, peripheral neuropathy; LM, leptomenigeal amyloid; Heart; cardiomyopathy.

Amongst the TTR mutations, some have been reported to appear as non-amyloidogenic (see Table 1.3). Frequency of each allele is varied in different populations. For examples, Gly6Ser is present about 12% of the Caucasian population, while, Thr119Met is found to about 0.8% of Portuguese and German populations. Interestingly, in some cases where both pathogenic and non-pathogenic mutations occurred, the non-pathogenic mutation apparently inhibited the development of FAP (Coelho *et al*., 1996)

Table 1.3. TTR non-amyloidogenic variants

| |
|---|
| Gly6Ser, Met13Ile, Asp74His, His90Ans, Gly101Ser, Pro102Arg, Arg104Cys, Arg104His, Ala109Thr, Ala109Val, Thr119Met, Pro125Ser |

### 2.3.2. Senile systemic amyloidosis (SSA)

Senile systemic amyloidosis (SSA) is the most common amyloidosis caused by the deposition of wild-type TTR (wtTTR) fibrils. It has been suggested being distinguished from TTR amyloidosis with variant TTR. It is the most prevalent systemic form of amyloidosis at least in the western world. Screening of Swedish and American above 80 years of age revealed that 25% to 28% of the people showed some degree of the disease (Westermark *et al.,* 1979; Cornwell et al., 1983; Westermark et al., 1990). However, it was rarely seen in individuals younger than 70 years (Röcken *et al.,* 1994). Pathogenesis of the TTR amyloid deposits are commonly found not only in heart, but also in lungs, blood vessels and the renal medulla of kidneys (Westermark *et al.*, 2003). SSA is typically manifested by cardiac disorders with congestive heart failure, arrhythmia and conduction blocks, and sometimes carpal tunnel syndrome.

The pathogenesis of SSA is unknown. There is no mutation in the TTR gene (Westermark *et al.*, 1990; Gustavsson *et al.*, 1995) and no evidence of over-expression of the TTR gene. On the other hand, the level of TTR in plasma slightly decreased in SSA (Westermark *et al.,* 1985). Most of the TTR molecules

found in the fibril of SSA are fragmented, leading to a postulation on an important role of fragmentation in pathogenesis of the SSA (Felding *et al.,* 1985; Westermark *et al.*, 1990; Westermark *et al*., 2003). The fragmentation of TTR molecule is not a random process because all of cleaved peptide bonds are situated within the C-strand, C-D loop and D-strand of the TTR polypeptide. However, it is not known whether the proteolytic cleavage plays a role in formation of TTR fibrils or merely represents a phenomenon of the post-amyloidogenic. Some post-translational modifications of wtTTR such as S-sulfonation and thiol-conjugation of the cysteine residue at position 10 of the TTR polypeptide have been implied to increase amyloidogensesis (Kishikawa *et al*., 1999; Suhr *et al*., 1999).

### 2.4. Fibrillogenesis of TTR

Several different forms of TTR have been suggested as precursors of the TTR amyloids. These include monomer, whole dimer, truncated dimer and tetramer forms of the protein (Lai *et al*., 1996; Lashuel *et al*., 1998; Schormann *et al*., 1998; Quintas *et al*., 1999; Eneqvist *et al*., 2000; Quintas *et al*., 2001; Serag *et al*., 2001). Several models of TTR amyloid formation have been proposed. However, in general, all of them were originated primarily on the formation that begins with the disruption of the protein tetramer into monomers (Figure 1.6). Tetramer has been believed separating into AB and CD dimers that further rapidly dissociate into monomers. Neither dissociation into AC- and BD-dimers nor sequential dissociation into monomers was suggested. These monomers are, then, undergo a conformational change, which promotes the formation of the amyloid fibrils (Figure 1.6) (McCutchen and Kelly, 1993; Lai *et al*., 1996; Kelly, 1998; Nettleton *et al*., 1998; Quintas *et al*., 1999; Jiang *et al*., 2001; Wiseman *et al*., 2005; Foss *et al*., 2005).

Three dimensional studies by X-ray crystallography of TTR structure suggested that several mutations of TTR polypeptide would destabilize the native tertiary structure of the protein. The largest change in the tertiary structure was observed in Leu55Pro. This finding is related to the clinical observation showing high tendency in amyloidogenic fibrillar forming of Leu55Pro (Sebastião *et al*., 1998). It was also shown that the mutations are predominantly situated in the hydrophobic core of TTR. The mutation “hot-spot” was usually located within C-strand, C-D loop and

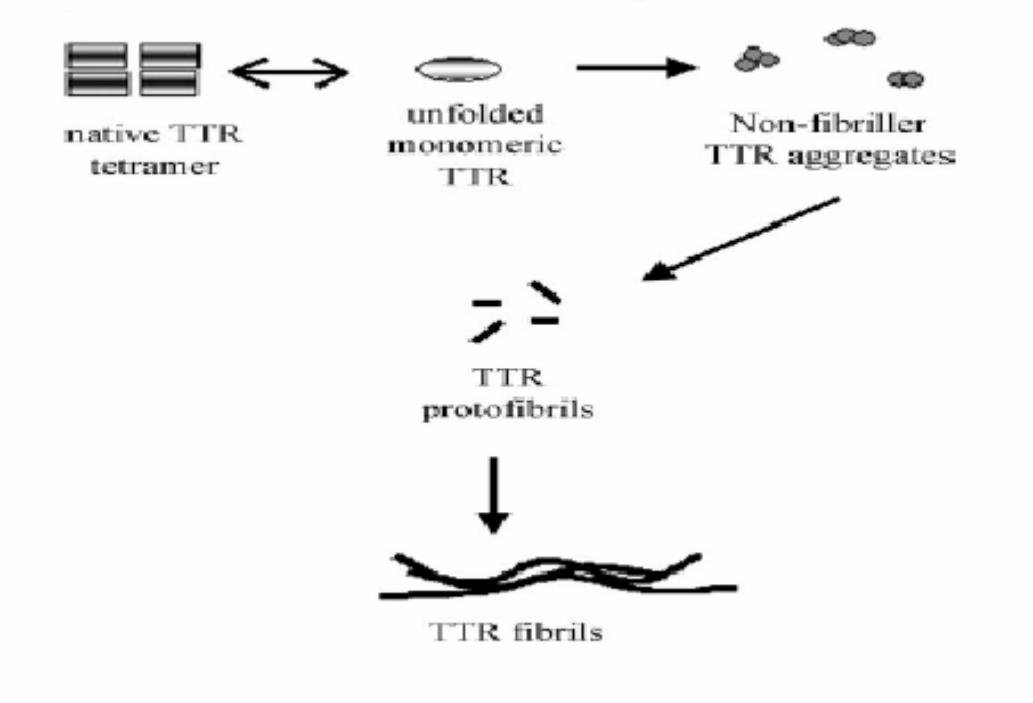


**Figure 1.6 The proposed pathway of TTR fibril formation.**
Native TTR tetramer dissociates into unfolded TTR monomers, which self-aggregated originating non-fibrillar TTR aggregates, protofibrils and mature amyloid fibrils. (From Sousa, 2003)

D-strand of each TTR monomer (Serpell *et al*., 1996; Eneqvist and Sauer-Eriksson., 2001), and has been suggested involving in the structural changes required for amyloid formation (Lai *et al*., 1996). Not all of TTR mutations lead to the disease. Some mutations were reported to increase stability of the tetrameric structure of TTR. The i*n vitro* studies on Val30Met/Thr119Met and Val30Met/Arg104His showed that these variants exhibited stability of the tetramer very close to that of the wtTTR (Almeida *et al*., 2000).

Although liver transplantation seems to be the only capable method of preventing progression of the disease, considerable risks for the patients and only slight improvements in neurological function are the limits (Lewis *et al.,* 1994). Less invasive therapies that target to reduce amount of amyloid in organs such as preventing the dissociation of TTR tetramer into monomers and disrupting the amyloid aggregates is being pursued (Figure 1.7). Several small molecules, such as tetracycline and T4, that show ability in binding to the thyroxine binding channel of TTR have been demonstrated to stabilize the TTR tetramer and inhibit dissociation into monomer species (Miroy *et al*., 1996; Peterson *et al*., 1998; Almeida *et al*., 2004). These molecules were demonstrated to inhibit oligomers of both wtTTR and TTR variants such as Val30Met and Leu55Pro *in vitro* (Miroy *et al*., 1996; Peterson *et al*., 1998; Almeida *et al*., 2004).

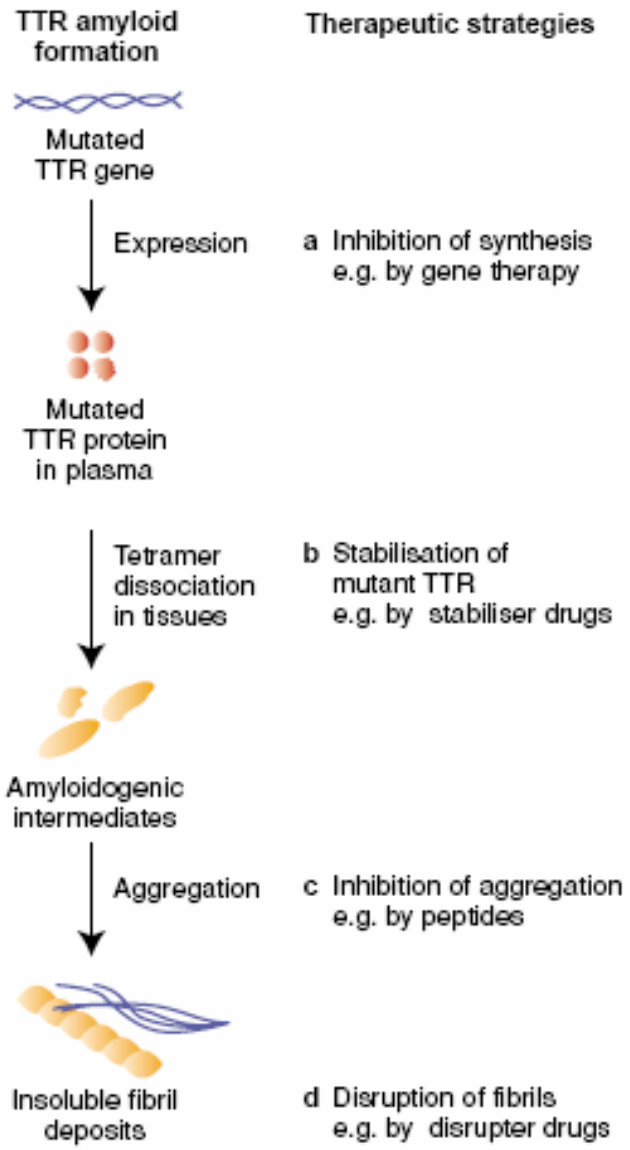


**Figure 1.7** **Potential molecular therapies for the TTR amyloidoses.**

Procedures that might block the amyloidogenic property of TTR include (a) inhibition of synthesis of mutated TTR, (b) stabilisation of the soluble circulating amyloid precursor, (c) inhibition of aggregation of amyloidogenic intermediates and (d) disruption of insoluble deposits (from Saraiva, 2002)

**3. Heterologous protein synthesis**

Recombinant DNA technology allows using foreign host cells either microorganisms, eukaryotes or plants for synthesis of heterologous proteins in sufficient amount for elucidating the structure and function relationship of the proteins. Several heterologous expression systems are now available including bacteria, mammalian cells, insect cells, and yeast. Each system has its own advantages and disadvantages (for reviews, see Verma *et al*., 1998; Terpe, 2006). Factors which need to be considered when choosing an expression system include biochemical and biological properties of the target proteins, levels of expression, requirement of posttranslational modifications and processing, and production costs (for reviews see Geisse *et al.,* 1996; Verma *et al.,* 1998).

Most of recombinant proteins are commonly expressed in *E. coli*. The most advantage of this system is that it can produce protein in a large amount. *E. coli* grows much faster than mammalian cells, and requires more general media than other host cells. This made *E. coli* expression system to be the cheapest. Another advantage is that transformation of foreign DNA into *E. coli* cells is easy and requires only small amount of the DNA. However, *E. coli* does not have a glycosylation, one of the important mechanisms of posttranslational modification of the eukaryotic proteins. Thus, it should be taken into consideration if the proteins of interest require such modification for folding and functions. In addition, the recombinant protein synthesized by *E. coli* has tendency to be trapped in an inclusion body. Therefore, refolding steps are needed to recover the active proteins.

There are several advantages of the heterologous protein synthesis system of yeast particularly in comparing to that of *E. coli*. Yeast is both microorganism and eukaryote, therefore, its genetics are more advanced than *E. coli* but it is easier to operate than mammalian cells. Yeast has an intrinsic ability of correcting expression, processing and folding of protein. It provides functional and fully folded heterologous proteins that can be secreted into culture media (Verma *et al*., 1998). Proteins that are insoluble as inclusion bodies when synthesized in *E. coli* are often synthesized as a soluble form in yeast. In addition, degradation of heterologous proteins, often a problem in *E. coli*, is usually reduced when synthesized in yeast. Unlike the mammalian cells, yeast can be rapidly grown on a simple growth

media. The terminating step of synthesis in yeast is similar to that found in higher eukaryotes involving termination of transcription and posttranslational modifications.

### 3.1. Heterologous protein synthesis in *Pichia pastoris*

*P. pastoris,* a methylotrophic yeast, have been recently developed as a host for heterologous gene expression. Promoter of *AOX1* gene that encodes for an alcohol oxidase (AOX), which is a key enzyme of the methanol pathway of *Pichia*, has been demonstrated to be a powerful tool for induction of the synthesis of the heterologous protein (Tschopp *et al*., 1987). Up to date, over 100 genes are placed and expressed in *P. pastoris* under the control of the *AOX1* promoter. Because the production of foreign proteins in *P. pastoris* occurs in simple minimal defined media, leading the system to a choice for NMR analysis of proteins that their folding can not recover from inclusion bodies or require post-translational modifications for proper folding or function (Wood and Komives, 1999). Two strategies have been used to integrate vectors into the *P. pastoris* genome, gene insertion and gene replacement (Cregg *et al.,* 1985). The *P. pastoris* transformant is usually initially grown on a repressing carbon source to generate biomass and then shifted to a medium containing methanol as the sole carbon and energy source to induce expression of the foreign gene. In the presence of methanol in the culture media, *AOX1* promoter is induced and the interested protein is expressed.

The most common *Pichia* that is used as a host for the expression is GS115 (*his4*). This *Pichia* strain is defective in *HIS4* gene that encodes for histidinol dehydrogenase. There are three phenotypes of *P. pastoris* expression strains regarding its methanol-utilizing ability i.e. methanol utilization positive ($Mut^+$), methanol utilization slow ($Mut^s$), and methanol utilization negative ($Mut^-$) (for review see Higgins and Cregg, 1998; Stratton *et al.,* 1998). Host strain and its methanol-utilizing phenotype are the most important factors to be considered when using the expression system (for review, see Cregg and Cereghino, 2000, Cregg *et al*., 2000). The wild-type strain of GS115 grows on methanol at wild-type rate ($Mut^+$) because its growth on methanol mainly relies on the presence of the *AOX1* gene. Another *Pichia* strain that frequent used as a host cell is a protease-deficient strain, SMD1168. This yeast

reduces a proteolytic degradation of foreign proteins very efficiently, however, their growth is usually slow and lower efficiency of transformation usually is obtained.

The heterologous proteins expressed in *P. pastoris* can remain intracellular after their synthesis. However, protein secretion into the medium is much more preferred as it is a major advantage for purification of the foreign protein product from proteins and other molecules of the yeast cell. Several different signal recognition sequences were used successfully in particular the signal sequence of *Saccharomyces cerevisiae* α-factor prepro peptide.

*P. pastoris* has more advantages in particular the type of glycosylation compared to other yeast strains. The N-linked oligosaccharides on several foreign glycoproteins secreted from the *P. pastoris* revealed a typical pattern of eukaryotic glycosylation (Montesino *et al.,* 1998). The majority of the N-linked oligosaccharide chains are rich in mannose, and the length of oligomannoside chain is much shorter in *P. pastoris* (Grinna and Tschopp, 1989). Glycans produced in *P. pastoris* do not have α-1,3-linked mannoside residues (Trimble *et al.,* 1991), which are responsible for the highly antigenic nature of glycoproteins used for therapeutic products (Cregg *et al.,* 1993). Furthermore, *Pichia* can grow at high cell density without accumulation of ethanol, which limits cell growth and the synthesis of the foreign protein, because respiration rather than fermentation is the preference of the expression process of *Pichia*.

### 3.2. Heterologous expression of TTR

Synthesis of recombinant TTR was first succeeded by using heterologous protein expression system of *E. coli* (Furuya *et al*., 1989; Murrell *et al*., 1992; Rosen *et al*., 1994). By using several bacterial expression vectors (Schoner *et al*. 1986; Furuya *et al*., 1989; Murrell *et al*., 1992), recombinant TTRs, either native or variant, with a subunit mass of 15 kDa could be produced. The recombinant protein was reported forming a tetramer with similar size as the native TTR (Furuya *et al*., 1989; Murrell *et al*., 1992) and could bind to T4 (Murrell *et al*., 1992). However, the N-terminus was reported having extra amino acid residues (Furuya *et al*., 1989) or still contained methionine that serves as the initiation site for translation in *E. coli* (Murrell *et al*., 1992). In addition, because *E. coli* secretes several endogenous

proteins, many steps are required to purify the recombinant protein from cell culture medium.

## Objectives

Screening for and characteristic study of TTR variants in Thai people.