Methods

1. Construction of expression vector for human wild type TTR and its variants

BamHI and EcoRI, or XhoI and EcoRI sites were introduced by polymerase chain reaction into human TTR cDNA such that cleavage by BamHI occurred immediately before the start codon ATG (for methionine) in the TTR cDNA presegment, by XhoI occurred immediately before the codon GGC of the first amino acid (Gly) of the mature TTR, and by EcoRI occurred immediately after the stop codon TGA of the cDNA. The PCR product with compatible restriction sites, BamHI at 5′ and EcoRI at 3′ end, or XhoI at 5′ and EcoRI at 3′ end, was ligated to *Pichia* expression vector pPIC 3.5 or pPIC 9.

TTR cDNA ligated to a *Pichia* expression vector was introduced into competent *E. coli* DH5α. Transformed *E. coli* cells were plated onto LB agar plates containing 100 μg/ml ampicillin. 10 ampicillin resistant transformants were inoculated into 2 ml LB medium with 100 μg/ml ampicillin and grown overnight at 37 °C. Isolation of plasmid DNA was carried out using the plasmid purification kit. Plasmid DNA was checked for the DNA insert by restriction analysis. Sequencing was performed to confirm the direction of the DNA insert in the plasmid, using 5′ *AOX1* primer (5′-GACTGGTTCCAATTGACAAGC-3′) and 3′*AOX1* primer (5′-GCAAATGGCATTCTGACATCC-3′). The DNA plasmid was linearized by digestion with *Sal*I at 37 °C for 2 to 3 h, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0.

2. Construction of human TTR variants

A site-direct mutagenesis and PCR were used to generate and amplify the cDNAs of human transthyretin variants, G6S, L55P, and V30G. The mutageneis was performed using the GeneEditor in vitro Site-Directed Mutageneis System of Promega. The reaction involved annealing of the selection oligonucleotide, encodes mutations that create a resistance to the antibiotic mixture which facilitates selection of the desired mutation, and the appropriate mutagenic oligonucleotide (Table 1) to the human TTR cDNA template. The mutant strand was synthesized with T4 DNA polymerase and T4 DNA ligase. The hetero-duplex DNA strands was then transformed into the BMH 71-18 *mut*S, a repair minus *E. coli* strain, and cells were

Table 1 Mutagenic oligonucleotide primers

TTR variant	primer sequence 5′→ 3′	altered codon	altered amino acid
Gly6Ser	GGGCACCAGTGAATCCAAGTG	GGT → AGT	Gly → Ser
Leu55Pro	AGTCTGGAGAGCCGCATGG	CTG → CCG	Leu → Pro
Val30Gly	TGTGGCCGGGCATGTGTTC	GTG → GGG	Val → Gly

grown in selective media. Plasmid was prepared from the transformants those are resistant to the antibiotic selection mix, and transformed into the JM109.

3. *Pichia* transformation

3.1 by an electroporation

To prepare *Pichia* cells for electroporation, a single colony of *Pichia pastoris* from a fresh yeast extract peptone dextrose (YPD) agar plate was grown in 5 ml of YPD in a McCartney bottle at 30 °C overnight. 100 µl of the overnight culture was inoculated in 250 ml of fresh medium in a 1-liter flask and grown overnight to an OD_{600} of 1 to 1.2. Cells were collected by centrifugation at 1500 x *g* for 5 min at 4 °C and resuspended with 250 ml of ice-cold water. Cells were collected and resuspended once more with 125 ml of water. Then cells were resuspended with 10 ml of ice-cold 1 M sorbitol and finally resuspended in 0.5 ml of ice-cold sorbitol to give a final volume of approximately 0.8 ml.

Electroporation was performed using an Electroporator II model of Invitrogen. 80µl of the *Pichia* cells was mixed with 10 to 20 µg of linearized DNA and transferred to a 0.2 cm electroporation cuvette. Cells were incubated on ice for 5 min and electroporation was carried out at a voltage of 1500 V, capacitance of 50 µF and resistance of 200 Ω, generating pulse lengths of ~10 millisecond with a field strength of ~7500 V/cm. Immediately thereafter, 1 ml of ice-cold 1 M sorbitol was added to the cuvette, the cells were flushed a couple of times and then transferred to a microfuge tube.

An aliquot of the cell suspension was plated on a minimal dextrose medium (MD) agar plate and incubated at 30 °C for 2 days.

3.2 by PEG1000 transformation method

Less, but sufficient, transformation efficiency of the expression plasmid into *Pichia pastoris* cells was also performed by the PEG1000 method, as described by the Invitrogen. In Brief, the competent *Pichia* was prepared by from the cells at early log phase (A600 of 0.6). Cells were collected and washed with solution contains 1 M Sorbitol, 10 mM Bicine and 3% (v/v) ethylene glycol. Cell suspension was aliquoted, and quickly frozen in the presence of DMSO. 50 µg of linearized TTR cDNA plasmid was added directly to a still-frozen tube of competent cells. The sample tube was

incubated at 37 °C for 5 min., prior to the 40% (w/v) PEG1000 in 0.2 M Bicine was added. The suspension was incubated at 30 oC for 1h. Then, cell was collected by centrifugation at 2000 xg for 10 min, and suspended in 10 mM Bicine, 0.15 M NaCl. Entire contents of cell suspension was spread on on a minimal dextrose medium (MD) agar plate and incubated at 30 °C for 3 days

4. Recombinant TTRs synthesis

4.1 Screening for Mut$^+$ transformants

Pichia transformants with phenotype His$^+$ Mut$^+$ were separated from His$^+$ Mut^s by growing the transformant mixture on minimal dextrose medium (MD) and minimal methanol medium (MM) agar plates. A colony of His$^+$ transformant from a MD plate was picked with a sterile toothpick and patched onto MM, then onto MD plate. Screening was performed with 50 to 100 His$^+$ transformants. Patches of GS 115/His$^+$ Mut^s albumin and GS 115/His$^+$ Mut$^+$ β-gal were included as His$^+$ Mut^s and positive controles, respectively. Plates were incubated at 30 °C for 2 days. The His$^+$ Mut$^+$ transformants grow very well in both MD and MM plate, while His$^+$Mut$^+$ transformants grow well in only MM plate.

4.2 Induction for gene expression of recombinant Pichia clones

The expression of the transthyretin genes were first carried out in a small scale. A single colony of His$^+$Mut$^+$ was inoculated into 5 ml of a glycerol or dextrose containing media in a 25 ml McCartney bottle. Cells were grown at 30 °C in an orbital shaking incubator (~200 to 220 rpm) for 18 h (OD_{600} of 2 to 6). Cells were collected, by centrifugation at 1500 x *g* for 5 min., and grown in 5 ml of methanol containing medium (OD_{600} of cell suspension at the start point of growing was 1) for 4 days. 25 µl of 100 % methanol was added every 24 h to maintain the level of methanol in the culture at about 0.5 %. Thereafter, the culture was centrifuged for 5 min and supernatant was analyzed for secreted proteins by silver stained SDS-PAGE.

5. Time course of TTR gene expression in yeast

Expression of a selected transformant clone was performed as described in Materials and Methods section 4.2. 100 µl aliquots of culture were removed after 24 h, 48 h, 72 h, and 96 h of methanol induction for determination of TTR content.

Culture samples were centrifuged at 1500 x *g* for 5 min at room temperature. Supernatant was collected and stored at –70 °C until assay.

6. Media selection

To compare the expression of the recombinant TTR gene in different media, an aliquot of colony of transformed *Pichia* was inoculated into BMG, MMGY BMGY or YPD (see composition of each media in Table 2). After overnight culturing, cells were transferred to BMY, MMMY BMMY or YPM and incubated for another 4 days. Thereafter, supernatant was collected and levels of expressed protein were analyzed by SDS-PAGE and protein bands were detected by silver staining.

7. Purification of recombinant human TTRs from yeast culture supernatant

7.1 by affinity chromatography

The recombinant human TTR was purified from the *Pichia* culture by an affinity column chromatography on human retinol-binding protein Sepharose4B according to Larsson et al (1985).

7.2 by preparative gel electrophoresis

Recombinant human TTRs were purified from the *Pichia* culture supernatant by preparative discontinuous Native-PAGE using the Prep Cell model 491 (Bio-Rad). The gel concentration (10% resolving gel and 3% stacking gel) and running conditions were optimized and performed according to the instruction manual of the company.

8. Determination of the mass of the TTR tetramer by gel filtration

The molecular mass of recombinant human TTR was estimated by FPLC/gel-permeation chromatography using Superose-12 column, equilibrated in 50mM potassium phosphate buffer saline pH7.4. 50µl (2 mg/ml) of purified TTR was chromatographed at a flow rate of 0.5ml/min. The column was calibrated with bovine serum albumin (68 kDa), ovalbumin (45 kDa), horse heart myosin (16.7 kDa) and horse heart cytochrome *c* (12 kDa).

Table 2 Compositions of *Pichia* media

Media	Compositions
BMG/BMY	100mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, 1% glycerol or 0.5% methanol
MMGY/MMMY	1.34% YNB, 1% glycerol, $4x10^{-5}$% biotin, 1% glycerol or 0.5% methanol
BMGY/BMMY	1% yeast extract, 2% peptone, 100mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, 1% glycerol or 0.5% methanol
YPD/YPM	1% yeast extract, 2% peptone, 2% glycerol or 0.5% methanol

9. Measurement of the thyroid hormone binding affinities of recombinant human TTR and analysis of binding data

The incubation of purified TTR (100 nM) with varied thyroid hormone, L-thyroxine (T4) or L-3,5,3′-triiodothyronine (T3), from 0-1000 nM was performed in the presence of tracer of ^{125}I-thyroxine or ^{125}I-triiodothyronine at 4°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 T4 or T3 from the same volume of incubation mixture was separated from the TTR bound thyroid hormones by adsorption to a layer of methyl cellulose coated charcoal on a glass microfilter under constant suction pressure. The radioactivity was determined using an LKB 1270 Rackgamma II counter with an efficiency of 70%. Non-specific binding was extrapolated and other corrections were performed prior analyzed by Scatchard plot. The K_d values were derived from the slopes of the least squares linear regression lines.

10. Western analysis

Electrophoresis of purified recombinant TTR was performed in duplicate then proteins were transferred to nitrocellulose membrane overnight at 4°C. Protein bands on the membrane were determined by staining with Coomassie. TTR protein band was detected by immunodetection using ECL Western blotting detection reagents, products of Amersham. Antibodies used were rabbit polyclonal antibody against a mixture of human, wallaby and chicken TTRs as primary antibody and HRP-linked anti-Rabbit Ig as second antibody. Filter was exposed to Kodak XAR-5 film with intensified screen at room temperature for 10 min, and then developed immediately.

11. Expression scale up

For expression scale-up, the culture volume was increase using baffled flask, according to the guidelines provided by the company with some alterations. In brief, a single colony of transformant was inoculated into 5 ml of BMGY in a 250 ml flask. Cells were grown at 30 °C at shaking speed of 220 rpm for 16-18 h. Sub-culture was carried to 250 liter of BMGY in 1 liter baffled flask and grew until reached an A600 of 2-6. Cells were collected by centrifugation at room temperature, and re-suspended in 300 ml of BMMY in a 2-liter baffled flask or 3-liter flask to an A600 of 1.0. Flasks

were covered with 2 layers of sterile cheesecloth. 100% methanol was added, to maintain methanol concentration in the medium to 0.5%, every 24 h until finished induction period of 96 h.

12. SDS-PAGE

For analysis under denaturing condition, proteins were separated in vertical sodium dodecyl sulphate polyacrylamide slab gels (15% polyacrylamide) using a 4% polyacylamide stacking gel and the discontinuous buffer system of Laemmli and Favre (1973).

13. Preparation of L[^{125}I]-thyroxine

In order to remove free ^{125}I and other degradation products from the commercial L[^{125}I]-thyroxine, the ^{125}I-thyroxine was chromatographed through SepPak C-18 cartridge, a reversed phase column (Mendel *et al.*, 1989). The purification was checked by thin layer chromatography (Pardridge and Mietus, 1980).

14. Amyloid formation

Purified recombinant TTR (2mg/ml) was induced to convert to an amyloid fibril by incubating at room temperature in a 50 mM sodium acetate, pH 3.6, for 72 h.

15. Congo red binding

Amyloid fibrils prepared from recombinant human TTR variants were incubated with 7 μM Congo red in 50 mM Tris pH 7.5, 100 mM KCl and examined for shifts on absorbance spectra (400-600 nm).

16. Preparation of competent cells and DNA transformation

Competent cells were prepared from *E. coli* strain DH5α with a transformation efficiency of 10^6 to 10^7, using calcium chloride, as described by Cohen *et al.* (1972). A single colony was picked from colonies freshly grown overnight on Luria-Bertani (LB) agar plates and transferred into 100 ml of LB medium in a 1-litre flask and incubated at 37 °C until the OD_{600} reached 0.5 (2 to 3h). Cells were transferred to polypropylene tubes (50 ml), cooled to 0 °C and collected by centrifugation. All supernatants were removed and tubes were inverted for 1 to 2 min

to drain away traces of media. Cell pellets were then suspended in ice-cold 0.1 M calcium chloride (10 ml per 50 ml original culture) and cooled to 0 °C. After centrifugation and draining, cells were suspended in ice-cold 0.1 M calcium chloride (2 ml/50 ml original culture). The competent cells were stored at –70 °C (as glycerol stocks).

To transform cells with foreign DNA, 50 µl of competent cell suspension was mixed with the DNA in a polypropylene tube and stored on ice for 30 min. The tube was then transferred to a water bath of 42 °C and incubated for exactly 90 s without shaking. Then, cells were incubated at 37 °C for 45 min and plated onto LB agar plates.