Production of Recombinant Human Transthyretin with Biological Activities toward the Understanding of the Molecular Basis of Familial Amyloidotic Polyneuropathy (FAP)†

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ABSTRACT: Transthyretin (TTR) is a plasma protein interacting with thyroxine T4 and retinol binding protein (RBP). Several variants of TTR with single amino acid substitutions have been identified as the major components of the amyloid fibrils of familial amyloidotic polyneuropathy (FAP), a fetal, autosomal dominant genetic disease. The elucidation of the molecular nature of the variants distinct from that of the wild-type TTR is crucial for understanding the amyloidogenesis in FAP, but our understanding is very poor mainly because of the unavailability of pure variant TTRs. In the present study, we used an *Escherichia coli Omp*A secretion vector (Ghrayeb et al., 1984) and achieved an effective production of the variant TTRs related to FAP including Met-30, Ile-33, Ala-60, Tyr-77, Met-111, and Ile-122 types. The variant TTRs produced in this system were efficiently secreted to the culture media. The chemical analysis showed that the secreted TTR (Met-30 type) has the same N-terminus as the native one. IEF analyses also indicated that the secreted product is properly processed as assessed by its p*I*. Furthermore, the secreted TTR was shown to have biological activities, namely, the thyroxin binding activity and the ability to associate with retinol binding protein, indicating that the secreted TTR polypeptide is properly folded. The present work also demonstrated that the processing/secretion of the recombinant TTR molecules in *E. coli* was strongly affected by single amino acid substitutions.

Transthyretin (TTR, prealbumin)[1] is a plasma protein having the high-affinity binding sites for both thyroxine and plasma retinol binding protein, and may play some roles in plasma transport of thyroid hormone and retinol. In plasma, TTR is found in the form of a tetramer consisting of identical subunits, and its higher order structures have been defined by x-ray diffraction analysis (Blake et al., 1974, 1978). Some variant forms of TTR have been known as a major component of amyloid fibrils found in familial amyloidotic polyneuropathy (FAP), an autosomal dominant genetic diseases [see Benson and Wallace (1989) and Saraiva et al. (1987)]. Patients with the disorder are heterozygous for the mutant TTR gene (Sasaki et al., 1984) in most cases and carry both wild and variant TTR molecules in plasma, but the variants are predominantly accumulated in the amyloid fibrils. Furthermore, the variants with different amino acid substitutions cause different clinical features of FAP: the variant with Met at position 30 (Met-30) for type I FAP, the Ile-33 for Jewish type, the Ala-60 for Appalachian type, the Tyr-77 for Illinois/German type, the Ser-84 for type II, the Met-111 for Danish cardiac type, and Ile-122 for cardiomyopathy (Kanda et al., 1974; Dwulet et al., 1983; Tawara et al., 1983; Saraiva et al., 1983a; Nakazato et al., 1984; Wallace et al., 1986, 1988a,b; Husby et al., 1985; Gorevic et al., 1989). These observations indicated that the comparative analysis of the molecular nature of wild and variant TTRs is crucial for understanding the amyloidogenesis in FAP, but little has been clear mainly because no pure preparation of the variants has been available. At this moment, it seems difficult to purify the variant TTRs directly from the patients' plasma because all the patients except one Swedish sibling of type I FAP and one patient of senile systemic amyloidosis are heterozygous for the mutation. So, we have attempted to establish the system for production of the variant TTRs by recombinant DNA technology.

We previously suggested that an *Escherichia coli* secretion vector developed by Ghrayeb et al. (1984) is useful for an efficient production of the variant TTR molecules; about 5 mg/mL TTR was secreted in the media, as the tetrameric product, which was easily purified by a two-step procedure (Furuya et al., 1989). In the present work, we further develop the system to produce all the types of FAP-related TTR

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[1] Abbreviations: BSA, bovine serum albumin; FAP, familial amyloidotic polyneuropathy; IPTG, isopropyl β-D-thiogalactoside; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); RBP, retinol binding protein; SDS, sodium dodecyl sulfate; TTR, transthyretin.

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Table I: Oligonucleotides Used for This Study[a]

primers	sequence	restriction sites involved	amino acid substitutions
FP	CCAGTCACGACGTTGTAAA		
RP	CAGGAAACAGCTATGACCATG		
HF 19	TTCGCTACCGTAGCGCAGGCTGGTCCTACGGGCACCGGTGAA		
HF 12	TGCATGTGATCAGAAAG	*Bcl* I	^{84}Phe → Ile
HF 14	CTCCTCAGCTGTGAGCC	*Pvu* II	^{85}Thr → Ala
HF 13	CACCAAATATTACTGGA	*Sap* I	^{77}Ser → Tyr
HF 31	AATGGGGAGCTGCCAAGTG	*Alu* I	^{88}Ile → Ser
HF 34	CACTTGGCAGCTCCCCATT		
HF 35	TGCCGCCCTGATGAGCCCCTA	*Dde* I	^{111}Leu → Met
HF 36	TAGGGGCTCATCAGGGCGGCA		
HF 28	ACGGCTGTCATCACCAATC	*Mse* III	^{30}Val → Ile
HF 30	GATTGGTGATGACAGCCGT		

[a] Underlined nucleotides indicate the target sites.

variants having the same N-terminus (and probably C-terminus) as the native ones, and demonstrate that the recombinant TTR (Met-30) produced in this system has the thyroxin binding activity and the same ability to associate with retinol binding protein as the native one.

Materials and Methods.

Enzymes and Oligonucleotides. All restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I, and *Taq* DNA polymerase were purchased from Takara Shuzo (Kyoto, Japan) and Nippon Gene (Toyama, Japan). Deoxyoligonucleotides were prepared by an automated DNA synthesizer (ABI Model 380A).

DNA Manipulations. The general DNA manipulations were done as described by Maniatis et al. (1982). DNA sequencing was carried out according to a modified dideoxyribonucleic acid sequencing method (Hattori & Sakaki, 1986) using modified T7 DNA polymerase (United States Biochemical Corp.).

Site-Specific Mutagenesis. Site-specific mutagenesis was performed according to two different methods: the conventional method of Morinaga et al. (1984) and the method using the polymerase chain reaction (PCR) of Higuchi et al. (1988). The presence of the mutation and the absence of additional sequence change were confirmed by DNA sequencing of the entire cDNA. Oligonucleotides used for the sequencing and site-specific mutagenesis are summarized in Table I. As for the construction of pUTR-30, -33, -60, and -77, the conventional method was applied, and pUTR-84, -111, and -122 were constructed by the method of Higuchi et al. (1988). After the site-specific mutagenesis, all the plasmid DNAs were introduced into *E. coli* JM83. These cells are grown for overnight at 37 °C, and plasmid DNAs were extracted. Since the mutations generate new restriction sites (see Table I), the mutated plasmids were selected by restriction endonuclease digestion followed by pulse field polyacrylamide gel electrophoresis (PF-PAGE) (Ito & Sakaki, 1988), which enabled us to separate the circular plasmid (nonmutated DNA) from the linear one (mutated DNA). The gels were run at initial current of 230 mA with a switching interval of 30 s for 16–20 h at 10–15 °C in a simplified hexagonal apparatus for contour-clamped homogeneous electric field gel electrophoresis. The linearized DNA fragments were electrophoretically eluted from the gel, recircularized with T4 DNA ligase, and introduced into *E. coli* D1210 ($lacI^q$, lacY) (DeBore et al., 1983).

Preparation of Cell Extracts and SDS–Polyacrylamide Gel Electrophoresis. Bacteria carrying the recombinant plasmid were grown in M9 casamino acid medium containing 50 μg/mL ampicillin with vigorous shaking at 30 °C. When the density of the culture reached 50–100 Klett units (30 Klett units = ~2 × 10^8 cells/mL), isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM to induce the production of the recombinant TTR (Duffaud et al., 1987). The bacteria were further grown at 30 °C for an appropriate period. The bacterial culture (10 mL) was centrifuged, and cell pellets were resuspended in 850 μL of distilled water. Cells were disrupted by sonication for 15 s, 4 times, on ice with a Branson Model 200 sonifer, mixed with an equal volume of 2 × Tris–SDS solution [125 mM Tris-HCl (pH 6.8), 4% SDS, and 10% 2-mercaptoethanol], and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) after 2 min of boiling. On the other hand, the supernatant of the culture medium (8 mL) was dialyzed for 18 h against 1 mM Tris-HCl (pH 7.6) and concentrated with lyophilization. The samples was resuspended with 100 μL of distilled water, mixed with 100 μL of 2 × Tris–SDS solution, and subjected to SDS–PAGE as described above.

Immunological Analysis. After SDS–PAGE, the proteins were electrophoretically transferred onto a poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P, Millipore) (Matsudaira, 1987), and TTR was visualized by anti-human TTR antibody and a second antibody–alkaline phosphatase conjugate (ProtoBlot, Promega).

N-Terminal Sequence Determination. The proteins secreted in the media were separated by SDS–PAGE, electroblotted onto a PVDF membrane (Immobilon-P), and then stained with Coomassie blue to visualize the protein bands. The protein band of approximately 16 kDa was then excised from the PVDF membrane in order to be sequenced. Sequencing was carried out on an Applied Biosystems 470A gas-phase peptide sequencer equipped with an on-line pheylthiohydantion (PTH) analyzer. A trifluoroacetic acid activated glass fiber filter was coated with 3 mg of polybrene and precycled in the sequencer to remove contaminants. The PVDF membrane was positioned on top of the glass fiber filter in the assembled sample cartridge. Nine sequence cycles were performed using the standard 3RPTH program.

Isoelectric Focusing (IEF). IEF analysis of the Met-30 recombinant TTR was performed by applying approximately 100 μg of lyophilized culture medium to a native PAGE gel; in order to monitor TTR migration, a parallel sample of FAP serum (35 μL) containing bromophenol was also applied. With the electrophoretic conditions chosen, TTR comigrated with the dye bromophenol front (Saraiva et al., 1989). TTR-containing gel slices were placed on an IEF 8 M urea–polyacrylamide slab gel with a linear gradient from pH 4 to pH 6.5. The bands were visualized by Coomassie staining.

Thyroxine Binding Assay. The recombinant Met-30 TTR was purified by similar procedures previously described (Furuya et al., 1989), and the thyroxine (T4) binding assay was carried out essentially as described previously (Saraiva et al., 1984). L-[^{125}I]T4 was purchased from New England Nucelar (Boston, MA) (1250 μCi/μg specific activity). The assay mixture (100 μL) contained 1.1 × 10^{-8} M recombinant TTR and 0.4 × 10^{-8} M L-[^{125}I]T4 (0.04 μCi/assay) in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 1 mM EDTA. A parallel assay mixture containing an excess (4 × 10^{-6} M) of unlabeled T4 was also investigated in order to examine displacement of labeled T4 from specific binding sites. After incubation for 30 min at 30 °C, the tubes were chilled to 0 °C, and protein-bound [^{125}I]T4 was isolated from unbound hormone by gel filtration on minicolumns of Sephadex G-25; free hormone bound to the gel matrix was eluted with 0.25 N NaOH.

Retinol Binding Protein (RBP) Assay. RBP binding to the secreted Met-30 recombinant TTR was investigated by ligand blotting. The proteins of the culture medium were separated by SDS–PAGE and transferred to a nitrocellulose filter. After being blocked with 5% bovine serum albumin (BSA), the filter was incubated with human serum RBP (200 μg/μL dissolved in 0.1% BSA), containing bound retinol, as assessed by its green fluorescence. RBP was isolated from human serum following previous procedures (Saraiva et al., 1983b). Visualization of RBP binding to TTR was performed following incubation with an anti-human RBP antibody (developed in rabbits against the RBP preparation) and second biotinylated antibodies (Amersham).

Results

Construction of the Met-30 TTR Expression Plasmids. We previously constructed plasmid pINTR-5 for production of Met-30 TTR (Furuya et al., 1989), but the TTR molecule produced by the system had seven extra amino acid residues at the N-terminus which were derived from polylinker sequence of the original vector (pIN III-113-*Omp*A1). We thus attempted to construct a plasmid for producing the complete form of Met-30 TTR. For technical reasons, the TTR cDNA sequence in pINTR-5 (the *Xba*I–*Hin*dIII 513 bp fragment) was once transferred to pUC19 as shown in Figure 1, and then the 21 bp sequence encoding the extra seven amino acid residues at the N-terminus was deleted by site-directed mutagenesis using the oligonucleotide HF 19 (Table I). The resultant TTR cDNA was reintroduced into the pIN III-113-*Omp*A1 vector (Ghrayeb et al., 1984) to generate pINTR-30 (Figure 1).

E. coli carrying the plasmid was grown at 30 °C and induced by isopropyl β-D-thiogalactoside (IPTG). At various times after induction, the products were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The protein bands of molecular weights ~18K and ~16K were inducibly increased in the cell extracts, but in the culture media (the supernatant after centrifugation of the culture), only the band of 16 kDa was observed (data not shown). The 16-kDa protein in the culture media was blotted by anti-human TTR antibody (data not shown). Since the sequence *Ala-Gly* at the C-terminus of the signal peptide is a target site of *E. coli* signal peptidase (Ghrayeb et al., 1984; Dalbøge et al., 1989), it was expected that the product of pINTR-30 was cleaved at the junction of the signal peptide and the TTR polypeptide. We thus considered that the 18-kDa protein is the plasmid-directed *Omp*A signal peptide–TTR fusion protein and the 16-kDa one is the processed product, namely, the monomer TTR polypeptide. The intensity of the 16-kDa band blotted by anti-human TTR antibody suggested that the yield of the secreted TTR by this newly constructed plasmid was similar to that (ca. 5 mg/L) obtained by our previous system (Furuya et al., 1989).

N-Terminus of the Secreted TTR. To confirm that the 16-kDa protein is the processed TTR monomer, the proteins in the pINTR-30 culture medium were subjected to SDS–PAGE, and the band of 16 kDa was transferred to a PVDF membrabe. The N-terminal amino acid sequence of the protein on the membrane was determined by a gas-phase peptide sequencer (ABI Model 470A). From the elution profiles, the first nine amino acid sequence of the protein was determined to be Gly-Pro-Thy-Gly-Thr-Gly-Glu-Ser-Lys, which is identical with that of native human plasma TTR (Kanda et al., 1974).

Isoelectric Focusing (IEF) Analysis. In addition to the N-terminal analysis, we also assessed whether the protein was

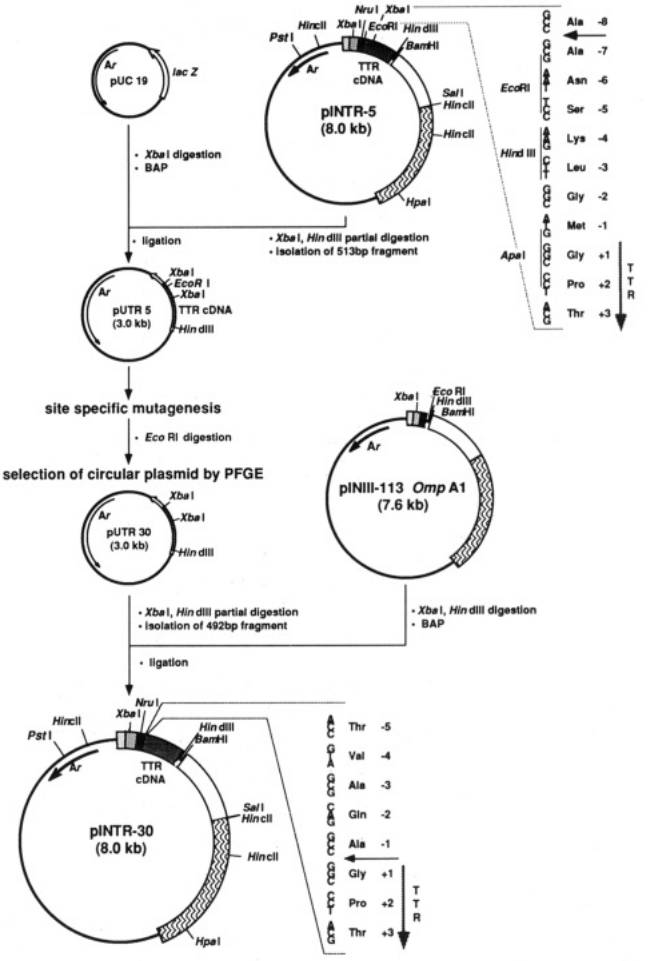

FIGURE 1: Scheme for the construction of the Met-30 TTR expression plasmid. The plasmid pINTR-5 (Furuya et al., 1989) encoding Met-30 TTR and seven extra amino acid residues was digested partially with *Xba*I and completely with *Hin*dIII, and the released fragment of 513 bp was ligated into *Xba*I-digested, bacterial alkaline phosphatase (BAP)-treated pUC 19 to get pUTR 5. To delete the 21 bp encoding the extra seven amino acids, site-specific mutagenesis was carried out using oligonucleotide HF19 (Table I) as described under Materials and Methods. The mutated plasmid (pUTR-30) was partially digested with *Xba*I–*Hin*dIII, and the mutated cDNA fragment (492 bp) was subcloned into the expression vector pIN III-113-*Omp*A1 (Ghrayeb et al., 1984). The plasmid was named pINTR-30. The promoter region is indicated by the stippled box and the *Omp*A signal peptide by the black box. The *lac* repressor (*lac* I) gene is indicated by the hatched box, Ar, β-lactamase gene. (→) Direction of transcription.

correctly processed by comparing the electrophoretic properties with those of TTR from a FAP serum by IEF analysis. Figure 2 shows the results obtained for this experiment. The recombinant TTR apparently focused at the same pH as serum TTR and also presented an oxidation product with the same p*I*. If an incomplete recombinant TTR was secreted, it would certainly produce a distinct IEF pattern (the last two C-terminal TTR residues are lysine and glutamic acid). Furthermore, these results indicate that differences in the primary structure of the secreted recombinant TTR and normal serum TTR are highly unlikely to occur. We thus concluded that the *Omp*A signal peptide–TTR (Met-30) fusion protein produced in this system was precisely and efficiently processed by the *E. coli* signal peptidase and monomer TTR was successfully secreted into the culture medium.

Thyroxine Binding Activity. TTR in plasma has the binding activity for thyroxine T4 and retinal binding protein (RBP). We at first asked whether the recombinant Met-30 TTR molecule showed the thyroxin binding activity. The recom-

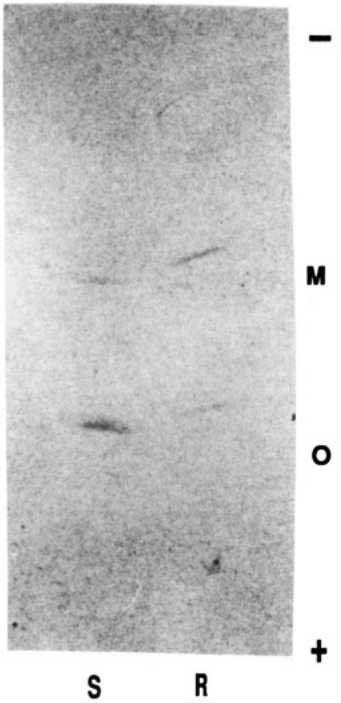

FIGURE 2: Isoelectric focusing of the recombinant Met-30 variant TTR. "S" is TTR from a FAP serum, and "R" is Met-30 recombinant TTR from the secretion medium. In addition to the TTR monomer (labeled "M"), an oxidation product (labeled "O") is observed. A slight distortion of bands is seen in this particular gel, but repeated analysis revealed it to be an artifact of the procedure.

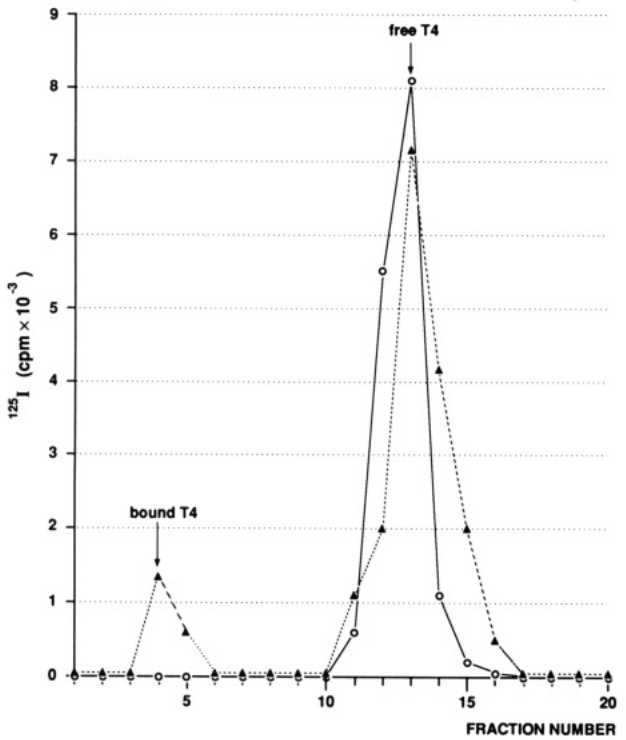

FIGURE 3: Elution profile of L-[^{125}I]thyroxine bound to recombinant TTR. The recombinant Met-30 TTR was incubated as described under Materials and Methods with (○) or without (▲) an excess amount of unlabeled T4, and the T4–recombinant TTR complex was separated from unbound (free) T4 on Sephadex G-25 at 4 °C.

binant TTR in the culture medium was partially purified, incubated with ^{125}I-labeled thyroxine T4 as described under Materials and Methods, and then subjected to gel filtration. As shown in Figure 3, a peak of radioactivity was observed at the position corresponding to that of the protein–hormone

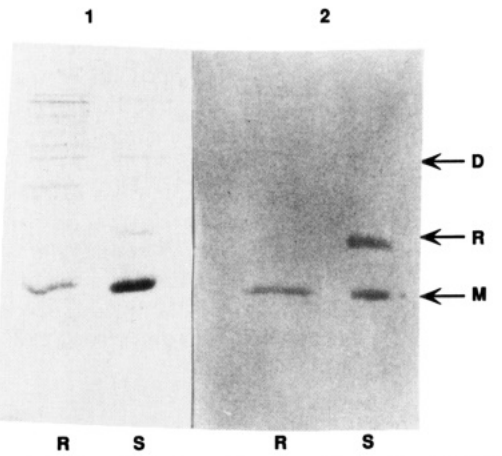

FIGURE 4: RBP ligand blotting. The recombinant Met-30 TTR from the culture medium (labeled "R" at the bottom) and a TTR–RBP standard (labeled "S") were analyzed by SDS–PAGE as shown on the left-hand side of the figure. Duplicate samples were transferred to nitrocellulose and incubated with RBP, and the TTR–RBP complex was visualized with an antibody specific for RBP as shown on the right-hand side of the figure. "M" and "D" refer to TTR monomer and dimer, 16 and 32 kDa, respectively. "R" at the right-hand edge refers to RBP (21 kDa).

complex. When the assay was carried out in the presence of an excess amount of cold T4, the protein-bound radioactivity completely disappeared. Although the assay was not quantitative, the results indicated that the recombinant Met-30 TTR is able to bind thyroxine T4. Native TTR forms tetramer in plasma, and our previous gel filtration assay showed that the secreted recombinant TTR having seven extra amino acid residues at the N-terminus very efficiently forms the tetramer (Furuya et al., 1989). The tetrameric structure is a prerequisite for the thyroxine binding activity (Blake et al., 1978). Thus, the results described above indicated that the recombinant (Met-30) TTR forms a tetrameric structure.

Association with Retinol Binding Protein (RBP). In order to investigate whether the recombinant TTR and RBP binding capacity as does normal serum TTR, the proteins from the culture medium were separated by SDS–PAGE and transferred to a nitrocellulose filter, and RBP binding was tested by ligand blotting. Figure 4 shows the results of these studies. For control purposes, a preparation containing the TTR–RBP complex, which dissociated with the electrophoretic procedure, was investigated in parallel. In the left-hand side of the figure, a protein stain analysis of this TTR–RBP preparation (marked S) and the culture medium (marked M) is shown. By ligand blotting, employing RBP and a RBP antibody to demonstrate RBP binding to TTR, a TTR band was visualized (shown in the right-hand side of the figure) for both preparations, i.e., for the TTR–RBP complex preparation and the secretion medium. Control experiments to rule out cross-reaction of the RBP antiserum with TTR were performed, demonstrating this antibody to be specific for RBP (not shown). These results imply that the recombinant TTR has the capacity to bind RBP.

Construction of the Expression Vectors for Other Variant TTRs. As described above, the expression system using *Omp*A signal peptide successfully produced the Met-30 TTR molecule having the same primary structure as the native one and also the biological activities. The system was further developed for producing other FAP-related TTR variants and wild-type TTR as well. Figure 5 presents a summary of the plasmid construction. The plasmid pUTR-RM having the wild-type TTR cDNA was first constructed from pUTR-30 and pUTR-6 (see Figure 1). The pUTR-RM was then mutagenized to

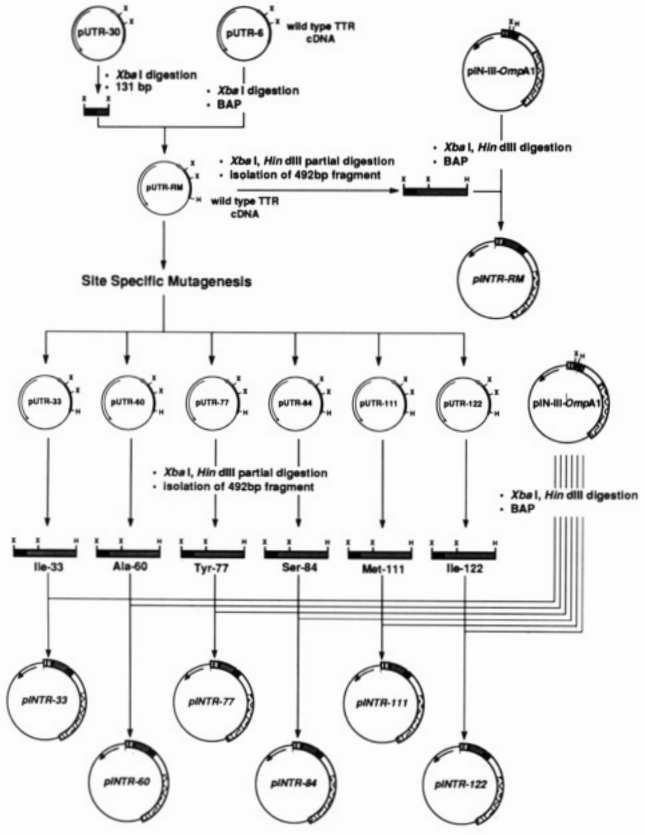

FIGURE 5: Scheme for construction of wild-type and variant TTR expression plasmids. The *Xba*I 131 bp fragment of pUTR-6, containing wild-type TTR cDNA (Sasaki et al., 1984), was replaced by the *Xba*I fragment of pUTR-30 (Figure 1). The resultant plasmid (pUTR-RM) was used as the source for the construction of other types of variant TTR cDNAs. The pUTR-RM was completely digested with the restriction enzyme *Hin*dIII and then partially with *Xba*I to release the 492 bp DNA fragment. This *Xba*I–*Hin*dIII fragment was introduced into *Xba*I and *Hin*dIII completely digested pINIII-113-*Omp*A1 to generate the expression plasmid of wild-type TTR (PINTR-RM), the other using pUTR-RM as a template. The mutagenized plasmids were digested partially with *Xba*I and completely with *Hin*dIII, and the 492 bp DNA fragments were isolated. The fragments were inserted into the *Xba*I–*Hin*dIII site of pINIII-113-*Omp*A1 to generate the expression plasmids. X; *Xba*I restriction site; H, *Hin*dIII restriction site, BAP, treatment with alkaline phosphatase to prevent self-annealing.

prepare pUTR-33 (Ile-33), pUTR-60 (Ala-60), pUTR-77 (Tyr-77), pUTR-84 (Ser-84), pUTR-111 (Met-111), and pUTR-122 (Ile-122). The *Xba*I–*Hin*dIII 492 bp fragments from these pUTR plasmids were introduced into the pINIII-113-*Omp*A1 vector, and the final TTR expression (secretion) plasmids (pINTR series) were obtained. When cells carrying these plasmids were grown in the presence of IPTG, two types of proteins (named "P" and "M", respectively) were inducibly produced and became the major cellular proteins at 3–12 h after induction (Figure 6). On the analogy of the case of the recombinant Met-30 TTR described above, it was considered that the larger protein ("P") of ~18 kDa is the *Omp*A signal peptide–TTR fusion protein, and the protein "M" of ~16 kDa is the processed recombinant TTR itself. This was supported by several findings: (1) "P" and "M" proteins were immunologically stained with anti-human TTR antibody by Western blotting (Figure 7); (2) the difference of molecular weights between "P" and "M" roughly corresponded to the size of the signal leader peptide (21 amino acids); (3) only the protein "M" was detected in the culture media (Figure 7); and (4) osmotic shock treatment failed to release the protein "P" into the media (data not shown). Thus, the TTR variants other than Met-30 type were also efficiently synthesized and secreted into the culture media by the *E. coli* OmpA vector system, although the yield of each variant has not been quntitated yet. These recombinant TTRs may be easily purified by a few steps of chromatography as described previously (Furuya et al., 1989).

It is interesting to note that the efficiency of the processing/secretion significantly differed from variant to variant (Figure 7). The Met-30 and Ile-33 variants showed the highest efficiency, but the Ile-122 variant showed very low efficiency. Unexpectedly, an extremely low amount (less than 1% of the Met-30 variant) of wild-type TTR was secreted. The wild-type clones were independently prepared for several times, and also several different *E. coli* strains were tested as host bacteria, but no efficient secretion was achieved. The variable processing/secretion efficiency may reflect the molecular nature of each TTR variant.

Discussion

In the present work, an expression system using *E. coli* secretion vector was constructed for the efficient production of human TTR variants related to FAP. The presented data strongly suggested that the recombinant TTR (Met-30 type) produced in this system was not only structurally but also functionally identical with or very similar to the native one.

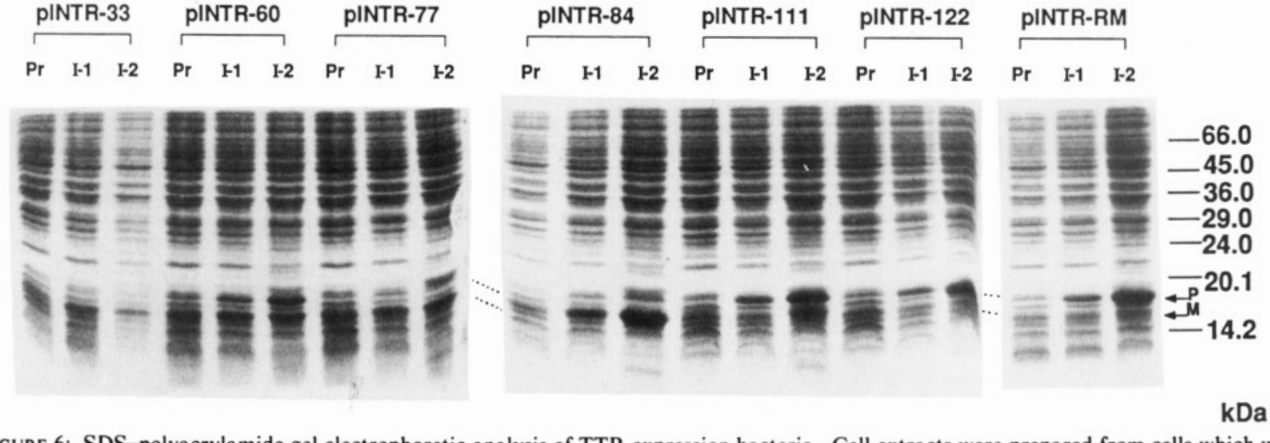

FIGURE 6: SDS–polyacrylamide gel electrophoretic analysis of TTR expression bacteria. Cell extracts were prepared from cells which were uninduced (lane Pr), induced for 3 h (I-1), and induced for 12 h (I-2), denatured, and subjected to SDS–PAGE as described under Materials and Methods. The proteins were visualized by staining with Coomassie Brilliant Blue. The protein bands corresponding to the molecular masses of monomer TTR (~16 kDa) and *Omp*A–TTR fusion protein (~18 kDa) are indicated by "M" and "P", respectively. Molecular mass marker proteins are bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.2 kDa).

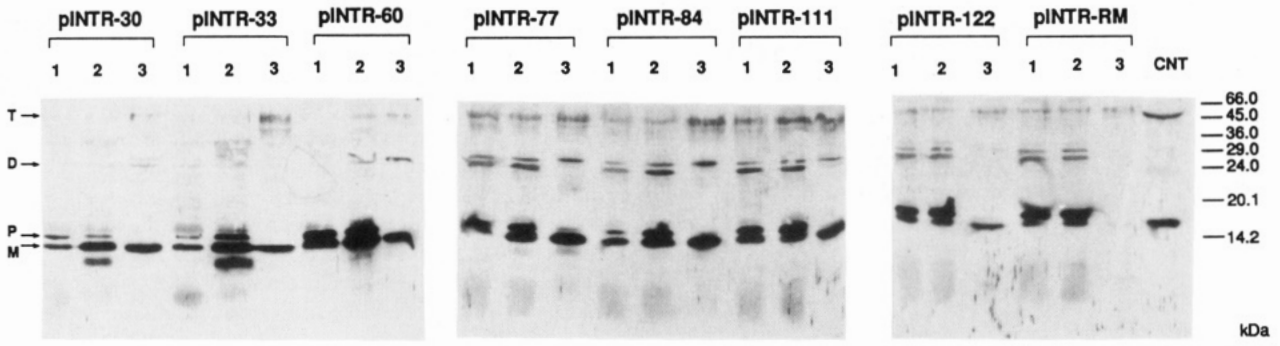

FIGURE 7: Immunoblot analysis of recombinant variant TTRs. Recombinant variant TTRs were subjected to SDS–PAGE. Proteins were transferred to the filter. The filter was treated by anti-human TTR antibody and a second antibody–alkaline phosphatase conjugate. For each variant: lane 1, extracts from cells without induction; lane 2, extracts from cells after 12-h induction; lane 3, culture media after 12-h induction. M, the position corresponding to monomer TTR; P, that to *Omp*A–TTR fusion protein; D, that to dimer TTR; T, that to tetramer TTR.

To our knowledge, this is the first system that enables us to produce the functionally active recombinant TTR.

To date, seven distinct variants of TTR have been identified as the major component of the amyloid fibrils in FAP, and the tight linkage between FAP and the variants has been genetically established [see Sakaki et al. (1989) for a review]. The variant TTRs are thus the primary cause of FAP, but FAPs with different types of variants are clinically different one another [see Benson and Wallace (1989)]. For example, the Met-30 variant causes peripheral polyneuropathy, autonomic neuropathy, and vitreous deposit of amyloid (type I FAP), whereas the Ala-60 variant, a mild polyneuropathy, bowel involvement, carpal tunnel syndrome, and cardiomyopathy (Appalachian type) and the Ile-122 only a senile cardiomyopathy. These findings indicate that the molecular nature of the variants is a crucial point to be analyzed for understanding the amyloidogenesis in FAP. However, little has been known about the characteristics of these variants mainly because no pure variant TTRs have been available. Almost all the FAP patients [except for a case of Swedish sibs homozygous for Met-30 (Holmgren et al., 1988) and a patient of senile systemic amyloidosis (Jacobson et al., 1990)] are heterozygous for the mutation, and their plasma contains both normal and variant TTRs. No method has been established to purify the variant form from the mixture of variant and normal TTRs. The TTR production system developed this present work may overcome this difficulty and enable us to produce any type of variant in a large amount without contamination of the wild-type TTR. The variant TTR molecules secreted into the culture media can be easily purified (Furuya et al., 1989).

In plasma, TTR forms the tetramer of four identical monomers and interacts with retinol binding protein (RBP) and thyroxine (T4). The secondary, tertiary, and quaternary structures of TTR molecule have been defined by X-ray diffraction (Blake et al., 1974, 1978), and possible amino acid residues interacting with T4 (Blake & Oatley, 1977) and RBP (Wallace et al., 1988a) have been proposed. The system described in the present paper may be valuable for studying the effects of FAP-related amino acid substitutions on the higher order structure of TTR as well as for verifying the structure and function relationship of the TTR molecule by introducing appropriate amino acid substitutions with the technique of site-specific mutagenesis.

Interestingly, the processing and secretion efficiency of the recombinant TTR molecules were considerably different one another. In particular, the wild-type TTR was synthesized normally, but processed very insufficiently and secreted at an extremely low efficiency. The present system might be also useful for understanding the molecular basis of the processing and secretion of the recombinant proteins produced in *E. coli*.

In conclusion, the TTR production system developed in the present work opened a new way to study the molecular basis of amyloid formation in FAP and also the structure–function relationship of the TTR molecule.

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Efficient and Selective Photoaffinity Labeling of the Estrogen Receptor Using Two Nonsteroidal Ligands That Embody Aryl Azide or Tetrafluoroaryl Azide Photoreactive Functions†

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ABSTRACT: 3-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophene **1** (tetrafluoroaryl azide, TFAA) and its protio analogue 3-(4-azidobenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophene **2** (protioaryl azide, PAA), photoaffinity labeling (PAL) reagents for the estrogen receptor (ER), have been prepared in high specific activity tritium-labeled form (19 Ci/mmol) and shown to undergo selective and efficient photocovalent attachment to ER from rat uterus. Both azides **1** and **2** demonstrate high binding affinity for ER as determined by both a competitive binding assay (relative binding affinities: estradiol = 100; TFAA = 9.3; PAA = 66) and a direct binding assay (K_d: estradiol = 0.24 nM; TFAA = 2.64 nM; PAA = 0.37 nM). When unlabeled TFAA and PAA are irradiated at >315 nm, they demonstrate site-specific photoinactivation of ER that reaches 43% and 55%, respectively, by 30 min. Specific photocovalent attachment to ER can be effected by irradiation of the tritium-labeled azides; the covalent attachment efficiency is good (**1** = 20–30%, **2** = ca. 25%) and the selectivity of ER labeling is high. Characterization of the photolabeled proteins by SDS–polyacrylamide gel electrophoresis shows specific labeling of a major component at M_r 60 000 and a minor species at M_r 46 000, the same two species that are labeled by [^{3}H]tamoxifen aziridine, a well-characterized affinity label for ER. The ER-specific antibodies H222Spγ and D547Spγ show a clean precipitation of only these two species. In the MCF-7 human breast cancer cell line, PAA is a full estrogen agonist in terms of stimulation of cell proliferation and induction of progesterone receptor. These two azides provide the first system in which the photocovalent attachment efficiency of an aryl azide can be compared to its tetrafluorosubstituted aryl azide analogue in a complex biological receptor system. Azides **1** and **2** are the most efficient and selective PAL reagents prepared to date for ER, and they should be useful in further studies of the hormone-binding domain of this protein.

Photoaffinity labeling (PAL)[1] reagents are versatile molecular probes for studying the ligand-binding domains of complex biological receptor systems (Bayley, 1983; Schuster et al., 1989; Tometsko & Richards, 1980; Gronemeyer, 1988). PAL reagents are especially valuable for mapping the contact points of ligands with proteins whose primary structure is known but

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[1] Abbreviations: ER, estrogen receptor; TFAA, tetrafluoroaryl azide; PAA, protioaryl azide; PAL, photoaffinity labeling; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; DATD, *N*,*N*′-diallyltartardiamide; RBA, relative binding affinity.

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