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Review

Transthyretin: a review from a structural perspective

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Abstract. Transthyretin (formerly called prealbumin) plays important physiological roles as a transporter of thyroxine and retinol-binding protein. X-ray structural studies have provided information on the active conformation of the protein and the site of binding of both ligands. Transthyretin is also one of the precursor proteins commonly found in amyloid deposits. Both wild-type and single-amino-acid-substituted variants have been identified in amyloid deposits, the variants being more amyloidogenic. Sequencing of the gene and the resulting production of a transgenic mouse model have resulted in progress toward solving the mechanism of amyloid formation and detecting the variant gene in individuals at risk.

Key words. Transthyretin; throxine; retinol; vitamin A; amyloid; structure.

Transthyretin: the historical foundation

Transthyretin is a plasma protein that began its structural life in scientific research in the laboratory of Dewitt Goodman where it was isolated and sequenced [1]. It was named prealbumin because it ran ahead of albumin on serum protein electrophoresis gels (this is true of the human but not the bovine protein). Goodman, who was primarily interested in retinol-binding protein (RBP), to which prealbumin complexes in vivo, and vitamin A metabolism, attempted to change the name prealbumin to transretin. Another researcher, Jacob Robbins [2], who made important contributions to the knowledge base concerning this protein, felt that the thyroxine-binding and transport properties of prealbumin needed to be emphasized in the name. Eventually, a compromise was reached with transthyretin [3].

Transthyretin has a monomer molecular weight of approximately 14,000 Da. It is produced primarily in the liver and excreted into the plasma [4, 5]. The newly synthesized protein has a 20-amino-acid signal peptide [1]. Transthyretin mRNA has also been found in kidney cells [6]. It has been shown by Dewitt Goodman and Xavier Roethig to make up a disproportionately large fraction (up to 25%) of the protein in the ventricular cerebrospinal fluid (CSF) [7, 8], although the concentration of transthyretin in CSF is lower than in plasma. This observation prompted the suggestion that transthyretin is either selectively transported across the blood-CSF barrier or synthesized de novo within the central nervous system (CNS). Joseph Herbert identified mRNA for transthyretin specifically and uniformly distributed in the cytoplasm and epithelial cells of the choroid plexus [7], a secretory structure located in the brain ventricles forming the blood-CSF barrier. Gerhard Schreiber found that, in rat brain, the choroid plexus contained 100 times the amount of transthyretin mRNA compared to the liver on

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a per gram basis [9]. Transthyretin has also been found in retinal pigment epithelium and in the pineal gland [10]. Adult pancreatic islet cells contain transthyretin [11] and the human fetus synthesizes prealbumin in the pancreatic islets of Langerhans and in the gastrointestinal mucosa [12]. In general, plasma transthyretin levels increase gradually after birth until they reach the adult concentration of 20–40 mg/dl, and then decrease after the fifth decade [13, 14]. Transthyretin is a negative acute-phase reactant, levels falling during conditions of malnutrition or chronic inflammation [15, 16]. The research group of Ingenbleek has intensively studied the nutritional aspects of transthyretin and its use as a nutritional parameter. A review of these studies has been published [17]. Transthyretin levels have also been reported to be unusually low in patients with transthyretin-associated amyloidosis (see below) and in a portion of at-risk individuals for this condition [18].

Human plasma transthyretin was crystallized in 1966 by Haupt and Heide [19] from ammonium sulfate solutions. The X-ray crystal structure of human transthyretin was determined in the laboratory of Colin Blake in 1971 [20]. It was shown to be a 55,000-Da tetramer with four identical subunits. In contrast to other three-dimensional structures of proteins known at that time, which consisted of high α-helical content or a mixture of α helices and β sheets, transthyretin has a very high β sheet content with one small length of α helix. Nearly all residues are involved in the β strands or the loops connecting them, or in the short α helix. Only the ten N-terminal and five C-terminal residues do not appear to be involved in the folding, and merely exist as a 'head' and 'tail.'

In future studies, the tertiary structure of transthyretin would provide a very important basis for hypotheses of the structure of the amyloid fibril and the mechanism of filbrillogenesis although, at the time the focus of the structural work was the binding site of thyroxine. Since the ability of transthyretin to form amyloid is a consequence of the structure, the important features are worth summarizing at this point. Blake and colleagues described the native human transthyretin structure in detail in a 1978 paper [21]. As in the case of hemoglobin, the tetramer is really two $\alpha\beta$ dimers related by a twofold crystallographic axis to give the tetramer. In the case of transthyretin, the monomer units in the $\alpha\beta$ dimer have identical sequence but slightly different conformation, particularly in the A-B loop region. The $\alpha\beta$ dimer consists of two monomer units, related by a pseudo twofold axis, whose contact region is an extended β sheet formed by extensive hydrogen bonding between the two individual β sheets which constitute the main structural component of each monomer. Approximately 45% of the amino acids of the transthyretin monomer are organized into a sheet of eight β strands, A–H, connected by loops, which result in a classic β barrel conformation. The β strands form an inner and outer β sheet, CBEF and DAGH, which are roughly orthogonal to one another and separated by about 10 Å. The strand names are primed in the β monomer (fig. 1). All strand interactions are antiparallel except between strands A and G. The single short α helix of nine residues comes after strand E. The extensive contact region between the monomers, formed by numerous hydrogen bonds, results in a strong dimer. The pseudo twofold axis relating the monomers is perpendicular to the surface of the sheets and passes between the H strand of one monomer and the H strand of the other. The β sheets are closer together in the vicinity of the α helix forming a compact structure and farther apart at the opposite end giving a more open structure. The open area is the result of a cluster of seven aromatic residues that probably stabilize the less compact main-chain conformation (fig. 2).

In contrast, the contact region between dimers, which produces the functional tetramer, is very small and consists of hydrophobic and hydrophilic interactions between the AB loop of one monomer and the H strands of the two primed monomers. The strength and extent of the monomer-monomer interactions suggest that the dimer rather than the monomer or tetramer is the basic unit in transthyretin structure. The idea of a dimer unit is supported by SDS-polyacrylamide gel (PAGE) analysis in which the dimer is still seen even in denaturing conditions [22]. The failure to observe subunits in SDS or urea was quite remarkable, although boiling for 5–10 min before running SDS-PAGE does show dissociation into monomers. Only by prolonged exposure to 6 M guanidinium chloride at pH 5.9, followed by dialysis against 5 M urea containing 0.1% SDS and then electrophoresis in 0.1% SDS was Robbins able to demonstrate the transthyretin monomer. These observations would later lead to the hypothesis that the dimer was the basic unit of fibrillogenesis. Despite the small contact area forming the tetramer, it is stable within a pH range of 3.5–12 and is not dissociated into subunits by strong acid. Another interesting feature of the tetramer observed by Robbins was the ability of the subunits of different transthyretin molecules to reassemble to form hybrid tetramers. This observation, together with the fact that most individuals are heterozygous for variants of transthyretin, delayed later structural studies on the transthyretin mutations. The transthyretin tetramer is not modified by phosphorylation, glycosylation, or acylation. One additional important residue, which featured prominently in later hypotheses of fibrillogenesis, is the lone cysteine at position 10.

Transthyretin and thyroxine transport

Transthyretin binds and transports 15–20% of serum thyroxine and up to 80% of thyroxine in the CNS [23].

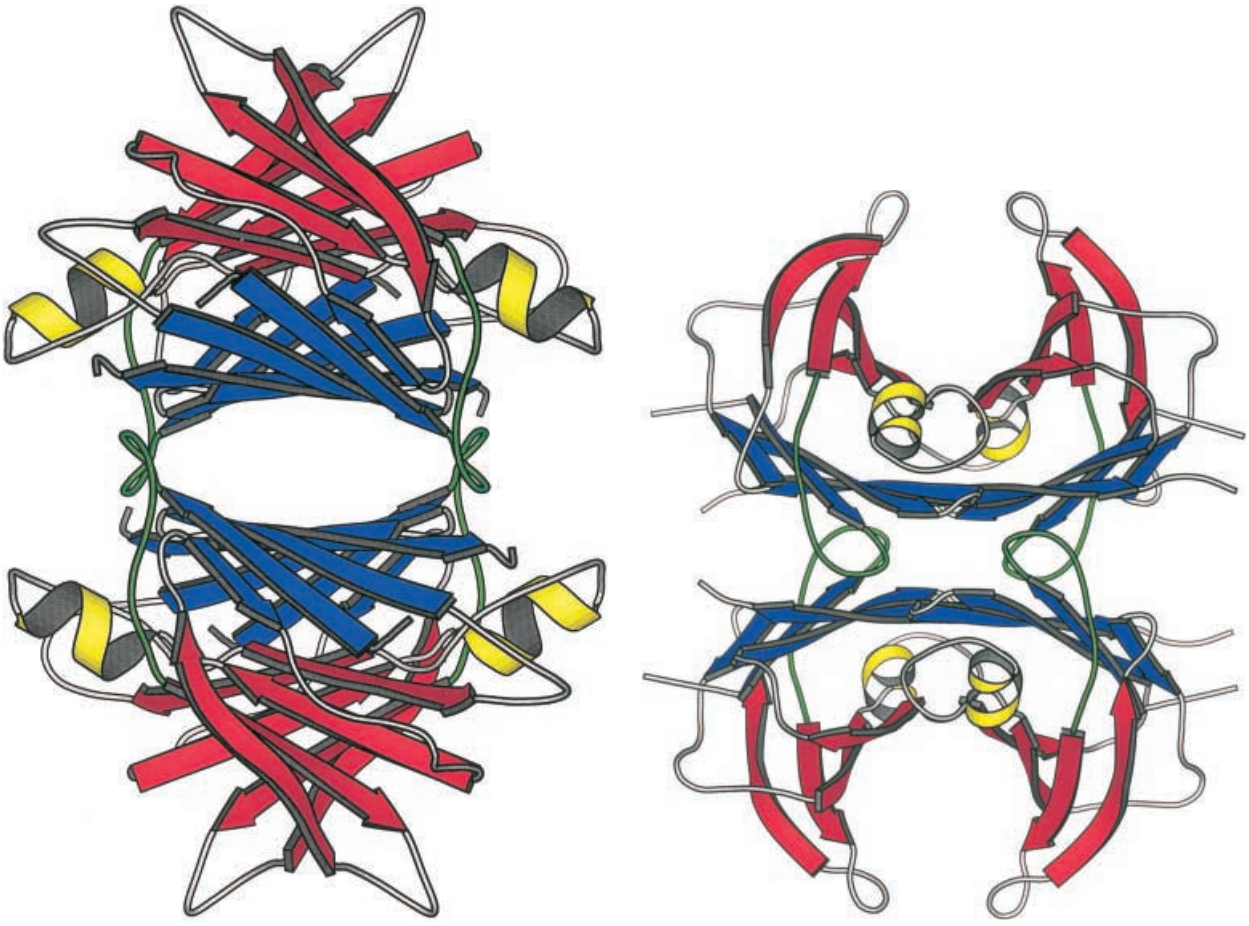

Figure 1. Ribbon drawing of the tetramer of human transthyretin. The extended inner β sheet (DAGH H′G′A′D′) is shown in blue, the outer (CBEF F′E′B′C′) in red. The lone helix is yellow, and the loops, which contribute to the formation of the functional tetramer, are in green. This drawing was produced by MOLSCRIPT [205] using coordinates from the Protein Database [206]. The file used is 1F41 [143].

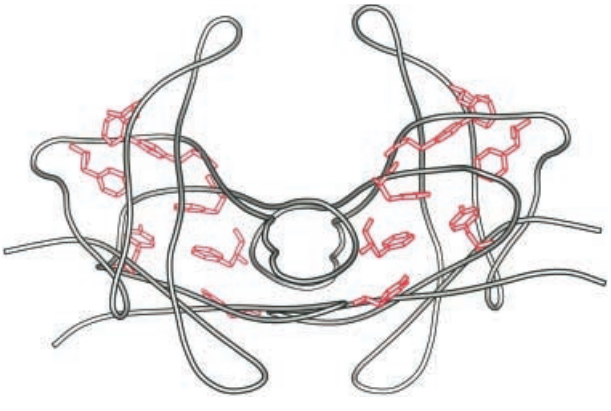

Figure 2. Ribbon tracing of the dimer of human transthyretin in gray with the aromatic residues between the extended β sheets shown in red. This drawing was produced by MOLSCRIPT from the same coordinates as in figure 1.

Other thyroxine-binding proteins in humans include albumin and thyroxine-binding globulin, the latter being the major serum transporter (75%). These proteins are responsible for delivering thyroxine to cells and keeping a large store of thyroxine in the plasma in a non-diffusable form. Free plasma thyroxine is in equilibrium with protein-bound thyroxine in plasma and protein-bound thyroxine in tissues. Non-protein-bound thyroxine is metabolically active [22].

Transthyretin mRNA is present in large amounts in the choroid plexus of all mammals, birds, and reptiles. In all cases investigated, expression of the transthyretin gene in brain was located exclusively in the choroid plexus [24]. The main function of the choroid plexus is the production of CSF but more recently, a number of plasma proteins have been shown to be synthesized by this tissue at high levels, including transthyretin [25]. The research group of Schreiber showed that choroid plexus transthyretin is secreted mainly into the CSF and not the blood, and that transthyretin in the CSF derives predominantly from the choroid plexus [26]. Blay and cowork-

ers [27] then proceeded to study the effects of thyroid hormones on transthyretin expression in choroid plexus by measuring transthyretin mRNA levels using Northern blots in hypo- and hyperthyroid rats. The distribution of radioactive thyroxine in the brain of these animals was studied after intravenous and intraventricular injection. While radioactive-labeled thyroxine could be seen distributed throughout the brain parenchyma after intravenous injection, labeling was confined to the cerebrospinal spaces after intraventricular administration. When protein synthesis was inhibited by cycloheximide treatment and labeled thyroxine was injected intravenously, the uptake of the labeled thyroxine in the choroid plexus decreased while that in the cerebral cortex increased. The authors concluded that this was indicative of thyroxine transport into the brain primarily through the blood-brain barrier and not via the choroid plexus and the CSF.

Transthyretin and thyroxine binding

Human serum transthyretin was the first of the major thyroxine transport proteins to be purified [2] and crystals were obtained in 1965 by Purdy et al. [28] and by Haupt and Heide [19]. The discovery by Goodman that transthyretin is also bound to RBP charged with retinol stimulated studies of transthyretin and the thyroxine complex as well as the complexation with RBP, and led to the seminal work of Blake and coworkers on the crystal structure of transthyretin [21, 29]. Early studies on thyroxine binding to transthyretin had concluded that there was a single thyroxine bound per transthyretin tetramer [30, 31]. The research groups of Ferguson and Cheng [32, 33] showed, using the fluorescent probe 8-anilinonapthalene-1-sulfonate, that there was one site with high affinity and a second site with lower affinity, and the fluorescent probe competed with thyroxine for the two sites on the transthyretin tetramer. Nilsson and his group in 1975 [31] used two fluorescent probes to examine the binding of thyroxine. The 8-anilinonapthalene-1-sulfonate bound to two sites, as had been observed previously, with an association constant of $3 \times 10^5\ M^{-1}$ whereas the 2-p-tobidinyl-napthalene-6-sulfonate only bound to one site, with a binding constant of $5 \times 10^5\ M^{-1}$. Both fluorescent probes were displaced from transthyretin by the addition of triiodothyronine and thyroxine, which had affinity constants of $9.6 \times 10^6\ M^{-1}$ and $9.3 \times 10^7\ M^{-1}$ respectively. These hormones bound only one site. 2-p-Tobidinyl-napthalene-6-sulfonate, triiodothyronine, and thyroxine, due to their size or orientation, were thought to sterically exclude binding to the second structurally identical site, i.e., a clear case of negative cooperativity. Regardless of the number of sites occupied, the tetramer conformation is necessary for thyroxine binding.

The structural identity of the thyroxine-binding sites on transthyretin was proved by Blake in 1977 through the crystal structure of the complex of transthyretin and thyroxine [34–36]. The thyroxine-binding sites are located in a channel (a β barrel of 16 strands) that runs through the center of the tetramer between two identical dimer units. The site is 50 Å long and 8 Å in diameter and completely envelops two molecules of the hormone. The chemical nature of the channel consists of three main elements. The first is a hydrophilic center that is formed from the hydroxyl groups of Ser 112, Ser 115, Ser 117, Thr 119, and associated bound water molecules. The side chains of Leu 110, Ser 115, and Ser 117 cause a constriction and narrowing of the channel near the mid-point. The second element is a hydrophobic patch formed by the methyl groups of Leu 17, Thr 106, Ala 108, Leu 110, and Val 121. A group of charged residues such as Lys 15, Glu 54, and His 56 constitute the third element at the entrance to the channel [29]. The two binding sites in the channel are identical by molecular symmetry. The negative cooperativity of hormone binding implies that only one molecule of thyroxine binds per transthyretin tetramer, and thus each site will have only half occupancy. Furthermore, the sites lie on a twofold axis of symmetry, so that each site itself has twofold symmetry. The crystallographic results therefore produced a weak and rather confused averaged image. As a result, the light atoms cannot be seen on the difference electron density maps and the only clearly defined features of the thyroxine molecule are the iodine atoms. Using the limited conformational freedom of the thyroxine molecule, fitting of a model structure was carried out using the iodine atoms as anchors. The results showed that thyroxine fits into the channel with each component of the hormone experiencing a favorable protein environment. The 3′ and 5′ iodine atoms make contact with the side chains of Leu 17 and Leu 110 and lie within the pocket lined with methyl groups. The 4′ hydroxyl group with the help of a water molecule interacts with the hydroxyl groups of Ser 117 and Thr 119. The thyroxine α-carboxylate and α-amino groups point outside the channel so that contact is made with the charged residues at the channel mouth (His 56, Lys 15, and Glu 54) [35, 36] (fig. 3).

The research group of Colin Blake re-evaluated the binding of thyroxine on the basis of structural studies of some additional thyroxine analogues [37]. This excellent and detailed review discusses the various possible binding modes of the hormone and its biologically important analogues. Thyroxine analogue studies showed that in a forward mode of binding, the phenolic hydroxyl group of the ligand interacts with residues Ser 117 and Thr 119 on transthyretin, either directly or via a bound water molecule. In the earlier model for thyroxine binding, the hydrogen-bonding potential of the phenolic group is not satisfied, despite spectroscopic evidence, which suggests

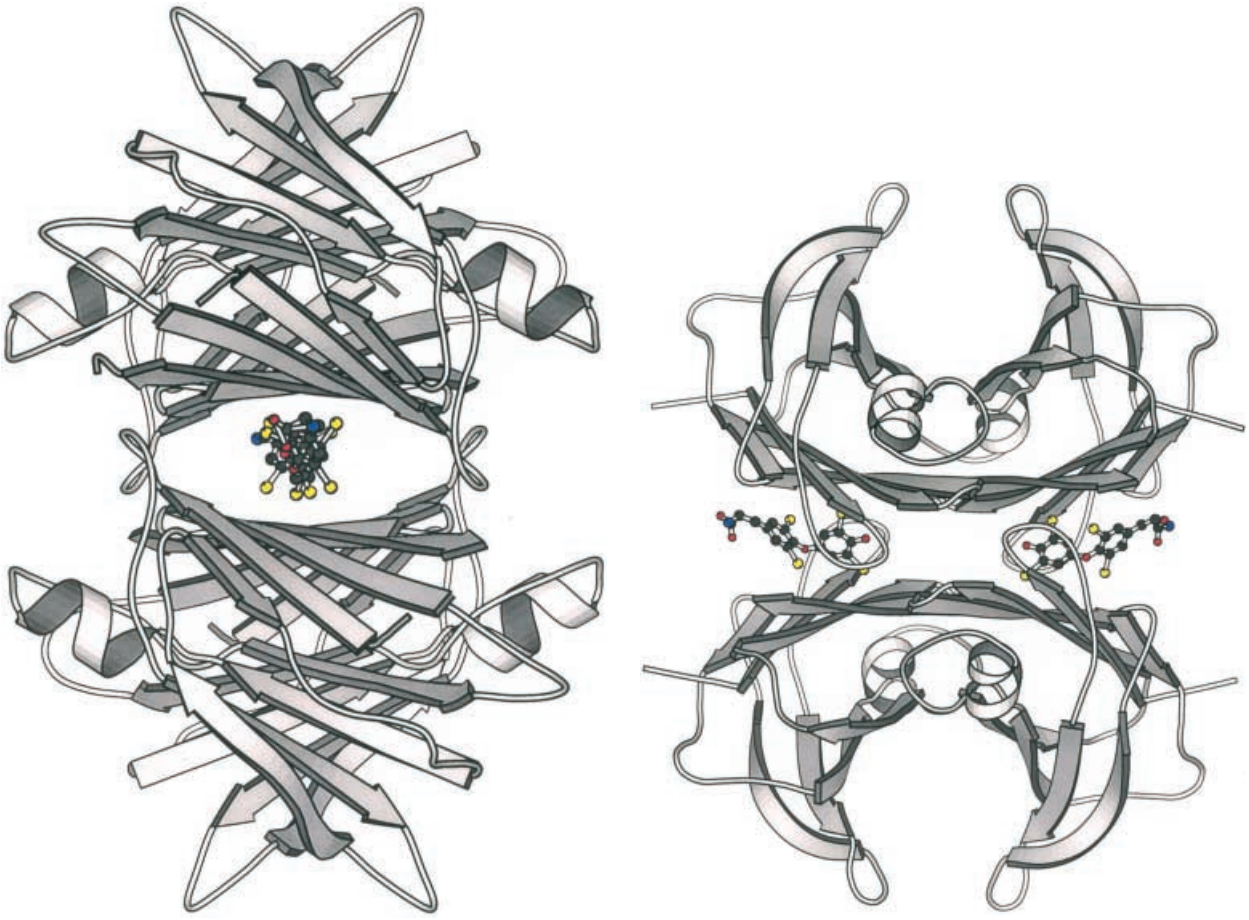

Figure 3. Ribbon tracing of the functional tetramer of human transthyretin in gray. The thyroxine molecule is shown in the two possible binding pockets between the dimers, although there is negative co-operativity of binding for the two positions. There are also other modes of binding for the thyroxine molecule that are not shown but discussed in the text. The carbon atoms are black, the oxygen atoms red, the nitrogen blue, and the iodine yellow. This drawing was produced by MOLSCRIPT using file 2ROX [59].

that this group is stabilized by the protein environment [32]. The structure of the diisopropyl-3,5-diiodothyronine complex showed that a compound constrained to adopt the same conformation as thyroxine can bind sufficiently close to the channel center to allow the hydrogen-bonding potential of the phenolic group to be satisfied. In addition, this can be achieved with 3′, 5′ substituents of approximately the same size and hydrophobicity as the iodine atoms. The observed position of the outer iodine peaks in the electron density maps of the diisopropyl-3,5-diiodothyronine complex coincided with those previously modeled for triiodothyronine in the 'distal' mode of entry where the hydrogen-bonding potential of the hydroxyl group is again satisfied. A refitting of the thyroxine molecule showed that the outer iodine atoms could be made to coincide more closely with those of diisopropyl-3,5-diiodothyronine while still being fully contained in the observed electron density peaks. This allows the hydrogen bonding requirements of the 4′-hydroxyl group to be satisfied without sacrificing any favorable interactions for the remaining substituents on the thyroxine, and indeed this new mode of binding can be accommodated without inducing any significant conformational changes in the Ser 117 and Thr 119 residues at the center of the binding pocket. However, this new model did not account fully for the observed thyroxine-induced conformational changes or the displaced positions of the outer iodine peaks in thyroxine relative to diisopropyl-3,5-diiodothyronine. Studies on 3′, 5′-diiodothyronine and reverse triiodothyronine showed them capable of binding in reversed mode. In both cases, displaced outer iodine peaks and also distinct conformational changes in the center of the binding pocket accompany this mode of binding. A model of reversed binding was produced for thyroxine which conforms to the necessary conformational constraints. The conclusion of these studies is that the structural data for triiodothyronine and thyroxine complexed to transthyretin can be best explained in terms of mixed modes of entry. In the case of triiodothyronine, a combination of 45% forward mode and 55% reverse mode or a variety of forward binding modes is possible. For thyroxine, there is also probably a combination of for-

ward and reverse mode but, at present, in unknown proportions.

Concurrent with the work of the research group of Colin Blake [37], structural studies designed to elucidate the mechanism of binding of thyroid hormones to transthyretin continued with the work of the research group of Vivian Cody. In 1992, this group published the structure of normal serum transthyretin with the thyroid hormone metabolite, 3,3′-diiodo-L-thyronine [38]. The overall transthyretin tetrameric structure was the same as that determined by Colin Blake. The two hormone-binding sites of the transthyretin tetramer were occupied by the metabolite. As in the case of Blake's complex of normal human transthyretin with thyroxine, the crystallographic symmetry produced an average of the two possible modes of ligand binding in the electron density map. Cody assigned 50% occupancy to each site in a statistically disordered model, as did the Blake group. The bound metabolite showed an overall transoid conformation with the ether bridge intermediate between skewed and perpendicular. The metabolite is bound 3.5 Å deeper in the channel (mode I) (called a translated forward binding mode by the Blake group), and with a different orientation than observed for thyroxine, thereby revealing the presence of another set of halogen-binding sites close to the center of the tetramer. Comparison with the thyroxine complex showed that the 3-iodine of the 3,3′-diiodo-L-thyronine occupies the same site as the 3′-iodine of thyroxine, and the metabolite 3′-iodine occupies the water site observed in the thyroxine complex. The binding affinity of the metabolite, 100-fold less than that of thyroxine, reflects the lack of the second pair of iodine atoms interacting in the pocket. Later in 1992, Cody's research group published another crystal structure of normal plasma transthyretin complexed with 3′, 5′dibromo-2′, 4,4′, 6-tetrahydroxyaurone [39]. This molecule, a flavone derivative, is a potent competitor for thyroid hormone binding to transthyretin as well as an inhibitor of iodothyronine deiodinase. Crystals of the complex were isomorphous to crystals of the complex with thyroxine and native transthyretin. Difference Fourier maps showed two binding modes for the guest molecules in each of two independent sites in the thyroxine-binding pocket. These were deep in the pocket near Ser 117 (mode I) and near the pocket entrance (mode II). None of the binding modes can be fully occupied because of overlap in the binding positions. Again, a statistical model was applied to account for the problems with crystallographic symmetry. The mode I and mode II binding sites were refined at half occupancy, resulting in two molecules per transthyretin tetramer. The bromoaurone binds in a non-planar antiskewed conformation. Comparison of the structural results with those of thyroxine indicated that the bromoaurone binding mode I is 3 Å deeper in the pocket and binding mode II is 4 Å farther from the channel center than thyroxine. These results are consistent with the conclusions of the Blake group, who suggest that 'the remarkable ability of transthyretin to accommodate ligands in a variety of binding modes may reflect its function as a transport protein for the thyroid hormones and their deiodination products, which under physiological conditions differ in their conformational flexibility at the ether link and in the charge properties of the phenolic hydroxyl group'.

Interest in naturally occurring variants of transthyretin increased due to the involvement of these point mutations in amyloid formation in patients with familial amyloidogenic polyneuropathy (see below), and several variants were discovered which showed altered binding of thyroxine. The affinity of transthyretin for thyroxine varies widely within the naturally occurring variants and does not appear to be related to the ability of the variant transthyretin to cause fibrillogenesis. In 1986, Refetoff and Benson [40] used sera from patients expressing several transthyretin mutations and reported that heterozygous forms of Thr60Ala transthyretin bind thyroxine with nearly normal affinity whereas heterozygous Val30Met and Ile84Ser mutant transthyretins bind thyroxine with reduced affinity. An additional variant was found in patients with familial euthyroid hyperthyroxinemia. In 1990, Alan Moses and coworkers [41] carried out sequencing studies on both the transthyretin gene and expressed protein in the proband of a kindred showing familial euthyroid hyperthyroximemia. The transthyretin in these individuals showed a threefold increase in thyroxine binding. Both DNA and protein sequences were consistent with a threonine for alanine substitution in amino acid residue 109 of transthyretin. All five family members were heterozygous for the mutation. Lalloz et al. in 1987 [42] also investigated a family where one individual showed an elevated thyroxine concentration. The individual inherited from her mother an albumin with an increased thyroxine-binding capability and from her father a transthyretin with an approximately fourfold increase in thyroxine-binding affinity. Fitch and colleagues [43] used PCR amplification and sequencing of the four exons of the transthyretin from the affected individual and revealed a heterozygous guanine to adenine point mutation at base 7 of exon 2. This results in a serine for glycine change at residue 6 of the mature transthyretin monomer. The individuals with these mutations showed no other abnormalities associated with transthyretin. There was no amyloid deposition and no reduced levels of RBP. Robbins group had shown in 1975 [32] that binding of thyroxine to transthyretin had no effect on the affinity of RBP for transthyretin. Due to the known ability of transthyretin dimers to form hybrid tetramers, to prove conclusively that the mutation was responsible for increased thyroxine binding, either a blood sample from a homozygous individual was required or the ability to make recombinant protein.

The ability to produce recombinant protein

The research group of Merrill Benson [44] hypothesized that these single amino acid changes affected the conformation of the transthyretin tetramer so that in some cases, β-amyloid fibril formation was enhanced and in others, binding to thyroxine and RBP was altered. The major obstacle to further structural work on the variants was the heterozygosity of the mutation. Transthyretin was known to form hybrid tetramers and a sample with uniform substitution was required. The research group of Merrill Benson solved this problem in 1992 [44]. As they stated in their paper describing the production of normal and variant recombinant transthyretin: 'Expression of mutant transthyretins in *E. coli* provides the opportunity to study structure/function relationships and amyloid-forming capabilities induced by single amino acid substitution in the transthyretin molecule'. The Benson group produced normal human transthyretin and five variant transthyretins: Gly6 $\rightarrow$ Ser, Leu58 $\rightarrow$ His, Thr60 $\rightarrow$ Ala, Ile84 $\rightarrow$ Ser, and Ala109 $\rightarrow$ Thr, using the pCZ11 expression system in *Escherichia coli* and site-directed mutagenesis. The recombinant transthyretin proteins showed the correct size on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western analysis and also self-associated into tetramers as determined by size exclusion chromatography. The tetramers were fully functional as thyroxine-binding proteins. The tetrameric structure is essential for thyroxine binding, since monomers and dimers are unable to produce the binding pocket. In competitive binding assays, normal recombinant transthyretin bound thyroxine with essentially the same affinity as normal transthyretin purified from human plasma.

A collaboration of the groups of Pedro Costa and Yoshiyuki Sakaki [45, 46] produced the Val30Met variant using the pIN-III-ompA-1 system, which resulted in a fusion protein with *E. coli* outer membrane protein A. The recombinant protein was secreted into the culture medium and formed tetramers. As with purified recombinant transthyretin from the other laboratories, their product showed normal binding of thyroxine and RBP, suggesting the proper tetrameric conformation had been produced.

Further studies on thyroxine binding

Refetoff and Benson [40] had previously demonstrated that native heterozygous Thr60Ala transthyretin bound thyroxine with an affinity not significantly different from normal transthyretin. Benson's group went on to show that the recombinant homozygous Thr60Ala transthyretin protein also retained its ability to bind thyroxine normally [47]. Studies on heterozygous native Ile84Ser, however, showed a significantly lower binding affinity for thyroxine than that of native normal transthyretin and homozygous Ile84Ser recombinant transthyretin bound thyroxine less well than native or the Thr60Ala recombinant transthyretin, suggesting similar effects on thyroxine binding in both the Ile84Ser homozygous and heterozygous proteins. Homozygous Ile84Ser had a relative binding of 0.41 to recombinant normal, whereas heterozygous Ile84Ser was 0.33 as compared with native transthyretin. Additional variants identified included Leu58His, Ser77Tyr, and homozygous Val122Ile, all of which showed decreased affinity for thyroxine [47]. Contrary to the variants described above, Leu58His and Val30Met transthyretin do not show the same binding affinity for thyroxine in the homozygous and heterozygous tetramer. Recombinant homozygous Leu58His transthyretin does not bind appreciable amounts of thyroxine. Its binding is significantly lower than that of the heterozygous form the variant. This result is rather surprising, since the Thr60Ala mutation does not appear to affect the binding of thyroxine, although it is only two amino acid residues away from the His58 residue. Heterozygous Val30Met transthyretin binds thyroxine with lower affinity than wild-type transthyretin, whereas homozygous Val30Met has almost no affinity for thyroxine. On examination of the structure of the transthyretin/thyroxine complex, none of the substituted residues in these variants are actually in the thyroxine-binding pocket. However, residue His 58 is close to the thyroxine-binding pocket and is near to His 56, which is involved in the binding of the α-carboxylate and amino group of thyroxine [48]. Substitution at position 58 may have a global effect on the conformation of the transthyretin tetramer, which is more significant in the homozygous state. Data for Ile84Ser, Val30Met, and Leu58His transthyretins suggest that amino acid substitutions in transthyretin can affect thyroxine binding even when they are located outside the thyroxine-binding pocket. One suggested explanation in the case of the Val30Met variant was that since residue 30 is internal, the increase in size from valine to methionine could alter the conformation, which could in turn affect monomer-monomer interactions and the formation of the transthyretin tetramer. This could then result in changes in the thyroxine-binding pocket. The Thr60Ala variant residue is located on the outside of the tetramer and does not interact with any of the structural elements forming the thyroxine-binding pocket. In this case, the amino acid residue itself could be affecting local interactions and binding but not changing overall conformation.

Another surprising result came from the data for thyroxine binding to the Gly6Ser variant transthyretin. Since this variant transthyretin was found in a family with euthyroid hyperthyroxinemia [43], this protein was postulated to have increased thyroxine binding. However, homozygous Gly6Ser recombinant transthyretin showed normal binding to thyroxine. These results might be ex-

plained by the assumption that only the heterozygous protein shows an increase in thyroxine affinity or that this variant does not have increased thyroxine affinity and another variant within the transthyretin gene is responsible for the observation in patients. Although the amino-terminal methionine residue in the recombinant protein could arguably cancel out the effects of the change at Ser 6, this is unlikely since the amino-terminal methionine residue does not affect normal thyroxine binding or the thyroxine affinity of other recombinant variants. Blakes's structural studies showed that the first ten residues of the transthyretin monomer were disordered, so the position of residue 6 with regard to the thyroxine-binding pocket is not known. From the work of Cheng et al. [33], the binding of N-bromoacetyl thyroxine to transthyretin showed that Gly 1, Lys 9, and Lys 15 were covalently bound to the thyroxine analogue. Blake had suggested that Gly 1 to Lys 9 is a flexible amino-terminal sequence positioned near the entrance to the binding channel. However, in a later, slightly higher resolution X-ray study, Hamilton et al. [49] showed that residue 6 was not involved in thyroxine binding.

In 1993, a group of researchers lead by Pedro Costa and Maria Saraiva identified individuals who had the transthyretin variant Thr119Met, which was associated with transient euthyroid hyperthyroxinemia [50]. In addition, they found an individual who was heterozygous for the double mutation Thr119Met and His90Asn. Serum dialysis with stepwise saturation of iodothyronine-binding sites confirmed that transthyretin binding of thyroxine was increased in the case of the Thr119Met transthyretin variant. They concluded that the observed increase in binding was due to a higher transthyretin concentration rather than an increased association constant for thyroxine. Since only a small proportion of serum thyroxine is bound by transthyretin, increased thyroxine binding did not affect the ratio of free to bound thyroxine or thyroxine concentrations. In contrast, the concentration of plasma RBP, almost all of which is bound by transthyretin, was elevated. The His90Asn mutation did not affect either the concentration of thyroxine or the affinity of binding to the variant. The Thr119Met mutation is interesting since, from the three-dimensional structure, it is located in the thyroxine-binding pocket, and is postulated to interact with both the 4′-hydroxyl group and the 5′-iodine of thyroxine. A substitution at this position might therefore be expected to alter thyroxine-binding affinity. In 1991, in the initial description of the Thr119Met variant by Harrison et al. [51], no increase in transthyretin binding of thyroxine was found after isoelectric focussing. In 1992, Scrimshaw et al. [52] examined the Thr119Met variant and found increased precipitation of both ^{125}I-thyroxine and ^{125}I-tri-iodothyronine by anti-transthyretin antibodies and postulated that the substitution resulted in an increased affinity of the Thr119Met transthyretin for thyroxine. However, after the publication of the results of the research group of Pedro Costa and Maria Saraiva described above, in 1994, the group set out to determine definitively whether the increase in the amount of thyroxine carried by the Thr119Met transthyretin was due to a change in affinity for thyroxine or to a change in thyroxine-binding capacity [53]. Their results showed that with normal serum, the resulting transthyretin isolated bound thyroxine with a single site of intermediate affinity ($K_a = 1.63 \pm 0.36 \times 10^7$ l/mol). No sites of higher or lower affinity were detected. Comparisons of binding capacity and immunoreactive transthyretin concentrations showed that the preparations bound thyroxine with a molar ratio between 1–2. With Thr119Met transthyretin, the thyroxine affinity was approximately doubled ($K_a = 3.40 \pm 0.76 \times 10^7$ l/mol; $P < 0.001$) with no change in immunoreactive transthyretin variant in the undiluted serum. This doubling in affinity explained the observed thyroxine levels of about 120 nmol/l in individuals with this mutation, which is identical to the value found by both Alves et al. [50] and Harrison et al. [51] in individuals with the Thr119Met transthyretin variant. Identical experiments carried out with Glu54Gly transthyretin showed that the thyroxine affinity of this variant was the same as for normal transthyretin. The conclusion of these authors, therefore, was that the Thr119Met transthyretin leads to secretion of a normal concentration of transthyretin protein that has a raised affinity for thyroxine. They suggest that substitution of a non-polar methionine for a polar threonine may allow the thyroxine molecule to interact more closely with the other amino acid residues in the binding pocket. They compared their results to the increase in affinity for thyroxine found by Moses and Benson for the Ala109Thr variant. The amino acid residue 54 in the Glu54Gly variant is farther away from the thyroxine-binding pocket and may therefore be unlikely to have an effect on thyroxine binding.

The plotting of the residues substituted in the many naturally occurring fibrillogenic variants on the three-dimensional transthyretin structure did not appear to form any coherent pattern that would suggest a reason for their fibrillogenic behavior, and, similarly, the substitutions that alter thyroxine affinity show no unifying pattern. In fact, amino acid substitutions on residues far removed from the thyroxine-binding pocket were found to alter thyroxine binding, for example Val30Met transthyretin and Ile84Ser transthyretin. To obtain more detailed information on the changes in conformation produced by the substitutions which result in altered thyroxine affinity, Hamilton and Benson began a crystal structure determination of homozygous Met 30 transthyretin (no observable binding affinity for thyroxine) and homozygous recombinant Ala109Thr transthyretin (increased binding affinity for thyroxine). The Val30Met transthyretin,

which was the first variant transthyretin to be identified with familial amyloidotic polyneuropathy and the first to be characterized [54–56], was isolated from the plasma of a patient who was homozygous for the variant [57, 58]. During the crystal structure analysis of the Val30Met variant transthyretin, the overall changes in conformation produced by the point mutation appeared to be small [49]. To minimize differences in conformation due to different experimental details such as resolution of the data, method of data measurement, and method of X-ray model refinement, crystals of normal plasma transthyretin were obtained and a structure determined and refined using the model of Colin Blake as a starting point and experimental procedures identical to those used for the variant structure [59]. The major differences between normal and Val30Met variant transthyretin consisted of displacement of the outer β sheet due to the increased size of the side chain of internal residue 30 and movements in the B-C, D-E, and F-G loops of both the A and B molecules of the transthyretin dimer. Thus the DAGH and CBEF β sheets are spread farther apart in the variant transthyretin. The movement is bell-shaped with the maximum change occurring opposite residue 30. Sheet DAGH, which, with its symmetry equivalent, lines the thyroxine-binding pocket, moves only slightly. Sheet CBEF shows the larger positional shifts and, since it is on the surface of the molecule, simply moves out farther into the solvent. Movements in the DAGH sheet, although small, change the shape of the central cavity that becomes more elliptical, with the major axis increasing by 0.4 Å and the minor axis by 0.3 Å. This may result in poorer fitting of the thyroxine molecule and explain the non-measurable binding of thyroxine to Val30Met transthyretin observed by Refetoff and Benson [40].

Cody hypothesized, on the basis of model building, that the substitution of Thr 109 by alanine in the Ala109Thr variant could cause repulsive interactions of the threonine side chain with the side chain of residue Leu 73 and attractive hydrophilic interactions could push the β strand in which it is situated toward the center of the binding pocket [39]. This would result in shortened distances between residues 108–110 and the hormone molecule, resulting in tighter binding of the hormone to the variant transthyretin. In 1993, the research group of Hamilton and Benson determined the crystal structure of the homozygous recombinant Ala109Thr transthyretin variant [60]. The results showed that the side chain of residue Thr 109 extends inward between the two extended β sheets. This was also true for the Val30Met variant. However, in the case of Thr 109, the extra atoms of the substituted side chain fit into empty space between the sheets and make no extensive changes to the molecular conformation. The substitution at position 109 causes small local changes in the secondary structure of the A, G, and H β strands resulting in a shift of residues 15 to 17, 108 to 110, and 117 in each monomer. The thyroxine-binding sites of the Thr 109 and Met 30 variants and of the normal transthyretin were compared, and the results suggest that the variation in affinity for thyroxine between the three proteins may arise from differences in the size of the binding pocket. The authors stated that it would be premature to suggest that the affinity for thyroxine is simply a function of width. However, the width of the binding pocket measured at the A β strand and also at the carbonyl oxygen of residue 109 (W_{AG}) correlates with the experimentally observed affinity of the three proteins for thyroxine:

$$W^{\text{Met 30}} < W^{\text{Norm}} < W^{\text{Thr 109}}; K^{\text{Met 30}} < K^{\text{Norm}} < K^{\text{Thr 109}}$$

Whether the static differences in the binding sites are relevant to the dynamics of the protein during thyroxine binding needs to be addressed by different methodology. To investigate the effect of the change in the position of Ser 117, the most significant difference in the binding site, on thyroxine binding, the authors solved the structure of the Ala109Thr variant of transthyretin complexed with thyroxine. This structure showed that thyroxine binding increases the width of the cavity at O109 and supported the conclusion that the position of O109 is an important factor in determining the affinity of transthyretin for thyroxine [unpublished results].

An additional unanswered question about thyroxine binding to transthyretin is that of negative co-operativity. In the current thyroxine-transthyretin model, the position of thyroxine in one site is not close enough to the second site to interfere directly with binding. A suggested explanation on the basis of the Ala109Thr transthyretin structure was that, since changes in the main chain torsion angles widen the binding site in the vicinity of the A β strand and narrow the binding site in the vicinity of the H β strand, there is a possibility that if thyroxine binding perturbs the positions of the A, G, and/or H β strands, those shifts may be transmitted to the second-site strands, A, G, and/or H β strands via the inter-strand hydrogen bonds, thus altering the affinity of the second site for thyroxine.

In 1992, Caroline Terry and Colin Blake [61] postulated a structure for a cleaved fragment of thyroxine-binding globulin by threading the sequence onto the crystal structure of cleaved α1-antitrypsin with which it shows high sequence homology. They were interested in structural features in the thyroxine-binding site, how these would relate to the thyroxine-binding pocket in transthyretin, and if the differences could explain the much higher affinity of thyroxine-binding globulin for thyroxine. They found that although these two thyroxine-binding proteins share no sequence homology and are otherwise different from one another, they have evolved to contain remarkably similar binding pockets for the hormone, which show the same overall shape, size, and chemistry. They appear to differ in one major aspect: the all-aliphatic binding site of the iodine substituent of thyroxine in

transthyretin is exchanged for a cluster of aromatic residues producing a predominantly aromatic binding site in the thyroxine-binding globulin. The authors hypothesize that these aromatic residues, which are absent in transthyretin, could account for the difference in thyroxine binding affinity displayed by the two proteins.

In 1995, the laboratory of Zanotti [62] determined the crystal structure of a complex of transthyretin with retinoic acid. The retinoid fits into the two chemically identical thyroxine-binding sites. The cyclohexene ring of the retinoid is innermost, occupying the same position as the phenolic ring of 3,3′-diiodo-L-thyronine, whereas the carboxylate group, as in the case of the thyroid hormone, participates in an ionic interaction with the Lys 15 side chain at the entrance to the thyroxine-binding channel. Although all-trans-retinoic acid was used to prepare the complex, the isoprene chain of the bound retinoid was found to be in a non-extended conformation. This allowed interaction of the carboxylate group of the retinoid with Lys 15. The authors concluded that conversion of all-trans-retinoic acid to cis-isomers had taken place to make this interaction possible. They hypothesized, on the basis of their findings, that the presence of a negatively charged group near an essentially hydrophobic core of the appropriate size was crucial for ligand-transthyretin recognition.

Transthyretin and RBP

In addition to binding and transporting thyroxine, transthyretin also transports retinol (vitamin A) from its main storage site in liver to target cells. The retinol is bound to RBP which, in turn, complexes with transthyretin. RBP has a molecular weight of 21,000 Da and would be rapidly eliminated from plasma by glomerular filtration in the kidney if it were not complexed to transthyretin. In addition, the transthyretin/RBP complex increases the affinity of RBP for retinol and shields retinol from the environment better than when retinol is bound to RBP alone [63]. This reduces the possibility of non-specific release of retinol from the retinol/RBP complex to acceptors other than the membranes of target cells. Initial investigations on the complex failed to delineate the precise location and number of sites for RBP on transthyretin. [64–66]

The crystal structure of RBP bound to retinol was determined by the laboratory of Alwyn Jones [67]. It is composed of an eight-stranded β barrel and a C-terminal helix and has a molecular mass of 21,000 Da. Retinol is encapsulated by the β barrel in the binding cavity in a 'hand-in-glove' fit with the ring end of the retinol innermost. Only the hydroxyl group of the retinol is solvent accessible. Apo-RBP has reduced affinity for transthyretin. The transition from apo- to holoprotein in human and bovine RBP involves a conformational change in the loop extending from residues 34 to 37, in particular Leu 35 and Phe 36. The space vacated by the removal of retinol in both proteins is filled by solvent molecules and the aromatic ring of Phe 36 [68, 69].

The involvement of Ile 84 of transthyretin in the complexation of RBP with transthyretin was postulated by Hamilton et al. [70] on the basis of a structure/function study of the variant transthyretin Ile84Ser. A study by the laboratory of Merrill Benson [71] had shown that individuals from a kindred with the amino acid substitution of serine for isoleucine at position 84 of the transthyretin molecule had substantially reduced plasma concentrations of RBP. In addition, when levels of RBP were measured in individuals from several kindreds with 17 different point mutations within the transthyretin gene, resulting in single amino acid substitutions, only those with substitutions at position 84 (Ser 84 and Asn 84) showed decreased plasma concentrations of RBP [72]. They concluded that the isoleucine at position 84 was important for mediating the binding of RBP to transthyretin. Hamilton et al. [70] determined the crystal structure of a homozygous Ile84Ser variant transthyretin. The major changes in conformation from the native protein were in the region of the mutation. Substitution at position 84 resulted in changes in the shape of the putative site for complexation with RBP. The proposed model for RBP complexation at this stage predicted that the C-terminal residues of RBP would be buried in the cleft between Ile 84 on one monomer and Ile 84 on a second monomer of the transthyretin dimer. The changes seen in the Ser 84 variant crystal structure suggested that substitution of the hydrophobic residue isoleucine by the more hydrophilic serine constituted the insertion of a hydrophilic residue into an essentially hydrophobic area. The serine residue at position 85 points out toward solvent whereas the isoleucine in the native transthyretin shields the tryptophan at position 79. As a consequence of the substitution, there is better solvent access to the neighboring residues. In addition Gly 83, Ser 85, and Pro 86 are pulled out of their positions in the native conformation. The importance of these residues of transthyretin in binding RBP was later proved by the crystal structure of the complex of the two proteins.

A crystal structure of a transthyretin/RBP complex, consisting of chicken RBP and human transthyretin, was determined to 3.1 Å resolution by the laboratory of Hugo Monaco [73]. This showed that the transthyretin tetramer was bound to two molecules of RBP (fig. 4). The two molecules of RBP formed inter-molecular contacts with the same transthyretin dimer (the 'so-called' same-dimer model), and each made additional contacts with one of the other two monomers. Thus, the other two possible binding sites for RBP on the transthyretin tetramer were blocked. The amino acid residues that were involved in the inter-molecular contacts were found to be close to the

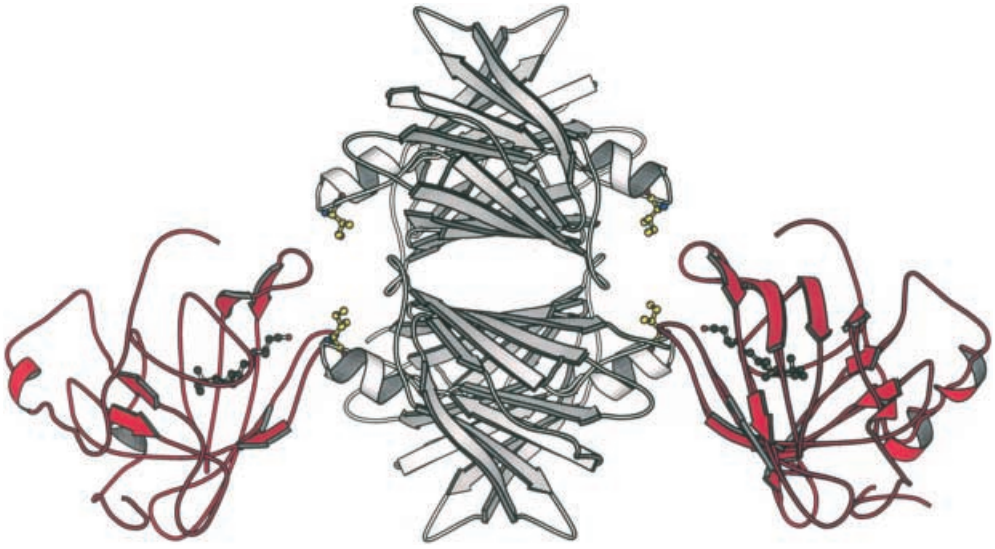

Figure 4. Ribbon tracing of human transthyretin in gray with the positions of residue 84 in yellow. The ribbon tracing of the two molecules of chicken retinol-binding protein complexed with the transthyretin are shown in red and the retinol molecule is black. This drawing was produced by MOLSCRIPT using file 1RLB [73].

retinol-binding site. Isoleucines at position 84 from two different chains of transthyretin participated in interactions with each of the RBP molecules, and the hydroxyl group of retinol was within hydrogen-bonding distance of the peptide carbonyl group of Gly 83 of the same transthyretin molecule that interacts with RBP Leu 35 and plays a role in the apo to holo transition. This could explain why apo-RBP has reduced affinity for transthyretin, since Zanotti et al. [68] had previously shown that for bovine RBP there was a conformational change involving residues 34–37 in going from the apo to the holo protein. In 1996, Margaret Sunde and coworkers [74] determined the crystal structure of chicken transthyretin to 3.0 Å resolution. Of the 31 residue differences found between chicken and human transthyretin, 9 occur at positions that, in human transthyretin, give rise to amyloidogenic variants, although none correspond to the appropriate side chain substitutions. The avian transthyretin is, for the most part, similar to the human protein except that the avian structure shows quite large differences in the region thought, from the work of Berni et al. [71], to be involved in the binding of chicken RBP to the human transthyretin. The chicken transthyretin structure shows loss of the short stretch of helix in the region of residues 75–89 and some differences in the monomer-monomer interactions. However, there is a sulfate ion situated near the distorted α helix that quite possibly resulted in the observed structural change. Chicken transthyretin binds thyroxine and the thyroxine-binding pocket is conserved in the avian structure. What effect would these differences have on the binding of RBP? What would this mean for the structure of the complex determined by the Monaco group?

The effect of the differences between human and avian transthyretin was resolved in 1999, by the research group of Marcia Newcomer [75] who determined the crystal structure of the complex of human RBP and human transthyretin to 3.2 Å resolution (fig. 5). Although the sequence of RBP is highly conserved, the chicken protein, which was used in the study by Monaco, lacks the eight amino acids at the carboxy terminus which are characteristic of RBP in all mammalian species, and a number of observations had suggested that there is an interaction of the carboxy terminus with transthyretin as discussed above. The laboratory of Rune Blomhoff [76] transfected COS cells that transiently expressed human and chicken RBP and used them to show that both proteins were able to bind retinol and human transthyretin. However, an increased retinol-dependent secretion was observed in cells expressing the chicken RBP and reduced ligand-dependent secretion with the human protein. They concluded that the C terminus, which is missing in the chicken RBP might play a role in retention and ligand-induced secretion. In addition, chicken transthyretin can bind up to four molecules of chicken RBP [77].

In the crystal structure of the all-human complex, RBP binds at a twofold axis of symmetry in the transthyretin tetramer, and the recognition site itself thus has twofold symmetry (the 'so-called' opposite-dimer model). Four transthyretin amino acids (Arg 21, Val 20, Leu 82, and Ile 84) are contributed from two monomers. Amino acids Trp 67, Phe 96, Leu 63, and Leu 97 from RBP are flanked by symmetry-related side chains in transthyretin. In addition, the C terminus of RBP, which is disordered in the structure of RBP alone, nestles in a hydrophobic patch at the interface of transthyretin and RBP, although it is only

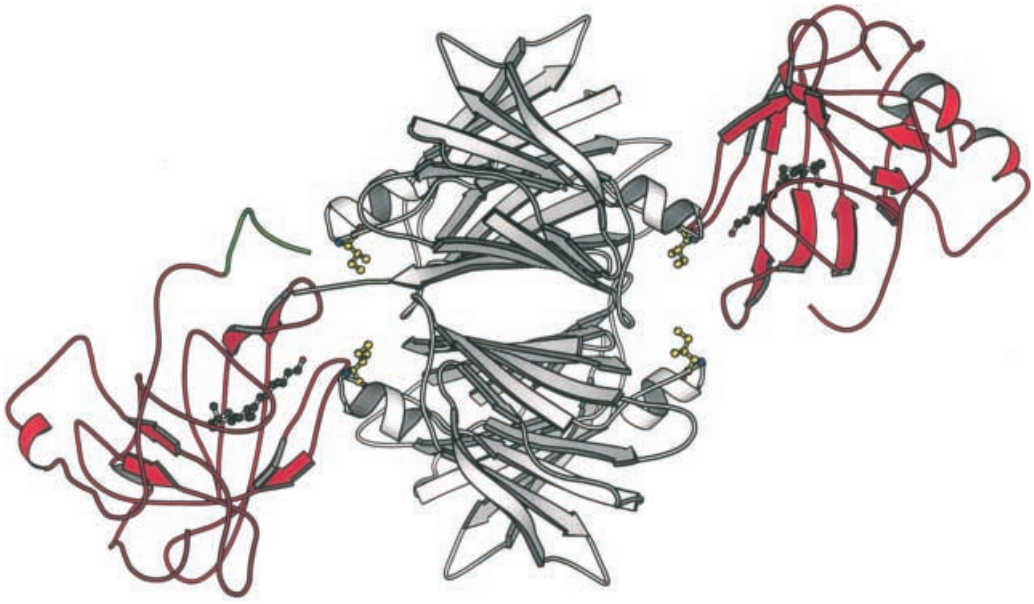

Figure 5. Ribbon tracing of the complex of human transthyretin with human retinol-binding protein. As in figure 4, the transthyretin tetramer is gray with residue 84 in yellow. The two retinol-binding protein molecules are red with the crucial tail involved in binding to the transthyretin in green and the retinol molecule in black. This C-terminal tail is not present in chicken retinol-binding protein. This drawing was produced by MOLSCRIPT from file 1QAB [75].

observed in one of the monomers. Substitution of Ile 84 by Ser or Asn would eliminate a significant portion of the hydrophobic core of the interaction surface and reduce binding affinity, as suggested by Hamilton. Support for a physiological significance of this interaction derives from the experiments on RBP clearance outlined above. In addition, Rask et al. [78] showed that human RBP exists in two forms, only one of which contains retinol, and this form is also the only one which can bind transthyretin. The retinol-free form does not complex with transthyretin. Previous studies had shown that the binding of retinol to RBP is not necessary for complexation with transthyretin. The two forms of RBP were sequenced and showed that the RBP which complexes retinol and transthyretin has an additional arginine residue at the carboxy terminus. The authors concluded that lack of this arginine causes loss of the ability to bind transthyretin.

Perhaps the 'same-dimer' model rather than the 'opposite-dimer' model may be a more favourable mode for the chicken protein and allow four molecules of RBP to complex when chicken transthyretin is used, as found in the studies of Kopelman et al. [77]. A recent review of transthyretin and RBP has been published by Hugo Monaco [79].

Variants of transthyretin and amyloidosis

Early studies

Amyloidosis is a name given to a group of deposition diseases in which insoluble deposits accumulate in tissues. These deposits are composed of homogeneous protein in the form of fibrils. The protein molecules aggregate into an ordered structure to make fibrils measuring 75–100 Å in cross-section and of indeterminate length [80]. The fibrils are resistant to proteolytic digestion and solubilization, rendering determination of the biochemical composition of the fibrils difficult. The ordered structure of the fibrils causes the deposits to be birefringent and, when histological sections are stained with Congo red, a characteristic green birefringence is seen by polarization microscopy [81]. The binding of Congo red to amyloid deposits is so strong that in patients treated with the dye for diagnostic purposes, the color is still visible at autopsy many years later [M. D. Benson, personal communication].

Until 1978 when Colin Blake published the refined structure of transthyretin and described its binding to thyroxine, the emphasis in research on transthyretin had focussed on the native structure and the consequent physiological functions, viz. binding RBP and transporting thyroxine. In that same year, Costa et al. [54] showed that amyloid material from patients with hereditary amyloidosis was composed of a subunit protein that reacted with antiserum to plasma transthyretin. This was confirmed by Skinner and Cohen in 1981 [82], and also at the structural

level by Merrill Benson in 1981 [55]. This finding had a major impact on the field of amyloidosis and on transthyretin studies. Research on transthyretin now proceeded in a different direction, to identify naturally occurring variants of transthyretin and their role in amyloidosis, to study the composition and structure of amyloid fibrils, to develop a theory of fibrillogenesis and, hopefully, to interfere with the process of amyloid deposition. To obtain fibrils for study, fibril concentrates were solubilized in 6 M guanidine-hydrochloride and fractionated on Sephadex G-100 columns. The eluted protein of apparent molecular weight 14,000 Da and antisera against the whole fibril concentrates reacted with the eluted protein and normal serum transthyretin. As a result of the harsh conditions required to solubilize the fibrils, it was impossible to tell if the transthyretin in the fibril was really a monomer or had been degraded by the solubilization procedure. The importance of transthyretin fragments and/or the whole molecule in fibrillogenesis is still undecided. Analysis of purified amyloid fibril proteins did lead directly to the definition of major classes of amyloid fibril proteins of unanticipated chemical diversity [83] and in some cases to the identification of the origin of the fibril protein precursor. One of the most important findings was the unifying definition of the amyloid fibril as having, in all cases, a β-pleated structure [84]. On the basis of morphological findings and studies with X-ray crystallography and infrared spectroscopy, a model was proposed incorporating a twisted β-pleated sheet fibril [85]. This became the basis for judging tissue deposits as amyloid. George Glenner [86] described the amyloid fibril as 'the abortive effort of the vertebrate to rival the silkworm' which 'represents the final common pathway for a diverse array of proteins in a variety of clinical conditions'.

The fibrils are composed of peptide chains that fold back and forth on themselves in regular intervals to form β sheets [80]. The adjacent chains are held 4.5 Å apart by hydrogen bonds. The side chain spacing between neighboring sheets is 9.8 Å. This anti-parallel β structure of amyloid allows for strong intra-strand stabilization, which gives amyloid its unique physical and chemical properties, such as insolubility and resistance to proteolysis. The pleated appearance described by Glenner et al. [85] is the result of residues not being fully extended but contracted so that the backbone of the structure is corrugated. The alpha carbon atoms lie along the creases in the sheet and the side chain atoms project alternatively above and below the plane of the sheet [87].

Familial amyloidotic polyneuropathy

Familial amyloidotic polyneuropathy (FAP) associated with variants of transthyretin represents the most frequent form of inherited amyloidosis [88]. In the United States, the prevalence of variant transthyretin genes may be as high as 1 in 100,000 [88]. The first published recognition of this form of amyloidosis was by Andrade in 1952 [89], and the first description of an American kindred by Falls et al. in 1955 [90]. In these syndromes, the amyloid fibril deposits contain the variant transthyretin and usually some normal transthyretin as well. FAP is not specific for the transthyretin variants, however. FAP can also be caused by a variant form of apolipoprotein A1 [91]. The possibility of other protein variants causing FAP must be considered. More recently, FAP has been divided into three classes on the basis of the clinical findings [88]. FAP-1 is usually associated with a variant form of plasma transthyretin. It is an autosomal dominant condition and most patients are heterozygous for the variant transthyretin, having one normal and one variant transthyretin allele. Expression of the two alleles is probably equal, but most studies show more of the normal gene product in the plasma than of the variant [92]. Patients homozygous for two different variants (methionine 30 and isoleucine 122) have been reported, but show no clinical features to distinguish them from their heterozygous fellow sufferers. There are now approximately 80 single-amino-acid variants of plasma transthyretin found to be associated with inherited amyloidosis [88, 93]. The majority of these are associated with neuropathy of the FAP-1 type. Amyloidosis is a disease of aging. The average age of onset of FAP-1 is 32–33 years, but with a considerable standard deviation.

The discovery by Costa et al. in 1978 [54] that transthyretin was a constituent of the fibril deposits in inherited amyloidosis began a concentrated effort to identify variant transthyretin carriers, which in turn required sequencing the transthyretin gene. A search was initiated for other variants and, simultaneously, efforts began to investigate the three-dimensional structure of the variants. These efforts were helped by new advances in molecular biology. In the case of heterozygotes, it was presumed, on the basis of recombination experiments in vitro, that the circulating transthyretin tetramer could be composed of a mixture of variant and normal transthyretin monomers in an unknown and perhaps variable proportion. This meant that isolation of the protein from plasma would not provide a sample of uniform chemical composition for X-ray structural analysis. Although the first X-ray crystal structure of an amyloidogenic transthyretin variant was carried out using protein isolated from the plasma of a patient homozygous for the mutation [59], this fortuitous situation could not be relied on and research moved in the direction of preparing recombinant protein. These efforts depended on work already carried out in sequencing the gene for transthyretin.

Beginning in the mid 1980s, the thrust of research on transthyretin covered a broad front. The discovery of variant transthyretin with altered thyroxine-binding capabil-

ity spurred attempts to determine in more detail the thyroxine-binding pocket and mechanism of binding. This was aided by molecular biology on the transthyretin gene and the ability to produce recombinant variant transthyretin. Research also progressed on the mechanism of control of the transthyretin gene, moving also into production of a transgenic mouse. This, in turn, allowed investigation into the mechanism of amyloid production. Research also progressed in the area of RBP, the structure of its complex with transthyretin and delineation of the binding site of the protein complex. Many more naturally occurring transthyretin variants were discovered but did not provide major help in producing models of the amyloid fibril or the mechanism of fibrillogenesis. Lastly, progress was made investigating the structure of amyloid fibrils using physical methods such as X-ray diffraction and high-resolution electron microscopy and a number of different models were proposed. In the clinical area, detection of gene carriers became possible although treatment of amyloid diseases has not made major progress to date. Supportive measures can significantly prolong life, and liver transplants remain the ultimate treatment of choice in the case of amyloidosis caused by transthyretin, but these are proving to be less than perfect solutions.

Sequencing the transthyretin gene

A second result that had a major impact on future research was the sequencing of the transthyretin gene. The human transthyretin cDNA sequence was reported in 1984 by two groups, those of Shuji Mita [94] and Yoshiyuki Sakaki [95] and in 1985 by Wallace et al. [96]. The complete structure of the transthyretin gene was reported in 1985 by Tsuzuki et al. [97] and Sakaki's group [98]. Using cloned human transthyretin cDNA as a probe, Southern blot hybridization of human genomic DNA revealed that the transthyretin gene consists of a unique single-copy DNA. The nucleotide sequence of the entire human transthyretin gene, including 581 base pairs of the 5′- and 95 base pairs of the 3′-flanking sequences was determined [97]. The gene has four exons and three introns and spans 7.6 kb. As in most eukaryotic genes, the consensus TATA and CAAT sequences are found 30 and 101 nucleotides, respectively, upstream from the putative cap site, and a polyadenylation signal sequence AATAAA is found in the 3′-untranslated region. Unexpectedly, two independent open reading frames, provided with respective regulatory sequences in their 5′- and 3′- flanking regions, were found within the gene, one in the first intron and the other in the third intron [97, 98]. It is not known whether these open reading frames are expressed in vivo. Tsuzuki et al. identified 300 [97] bp sequences, which were strikingly homologous to the human Alu-type repeat elements, and were shown by Sakaki and colleagues [98] to have opposite polarity. The single-copy transthyretin gene has been assigned to the long arm (q) of chromosome 18 [99]. The laboratories of both Simon and Niikawa further assigned the gene to the region 18q11.2–q12.1 [100, 101].

Another result that was to have a major impact was the sequencing of the transthyretin gene in mouse by the group of Robert Costa [102]. This would lead to efforts to produce a transgenic mouse model for amyloidosis. Glucocorticoid receptor-binding site sequences are found in the 5′ region of the mouse gene and in the first intron [103]. Another conserved sequence that resembles the enhancer element found in the immunoglobulin kappa chain gene is present in the 3′ end of the first intron. The –50 to ~ –190 region of the gene is highly conserved between rodents and humans [103, 104] and, in rodents, was shown to contain crucial elements for hepatocyte-specific expression, including the binding sites for HNF-1, C/E BP, HNF-3, and HNF-4 [103]. A tissue-specific enhancer has been identified in the –1.85 ~ –1.96 kb region of the mouse transthyretin gene, which functions in HepG2 cells but not in HeLa cells [103, 105]. The enhancer region contains the binding sites for AP-1, C/E BP, and HNF-4 [103]. The research group of Yoshi Sakaki performed a comparative analysis of the human and mouse sequences and showed that the binding sites for HNF-3 and HNF-4 were well conserved in the human transthyretin gene, but those for C/E BP were not as well conserved. The presence of a potential binding site for HNF-1 in the human transthyretin gene has been identified by Courtois et al. [106]. Binding sites for other liver-specific proteins, Tf-LF1, Tf-LF2, and LF-A1, were found by Sakaki at –216 ~ –221 and –199 ~ –204. Two long stretches of purine-pyrimidine dinucleotide sequence were also found which might be involved in the regulation of the transthyretin gene.

For brain expression, an additional transcriptional site is used, located at least 65 bp upstream from the normal start site [107, 108]. This site is not used in the liver, strongly implying that different transcriptional factors are used by each tissue. Extrahepatic transthyretin synthesis is unlikely to play a role in the systemic manifestations of amyloidosis, but occasional leptomeningeal involvement may be related to intracranial synthesis, and vitreous amyloid may possibly be the result of local gene expression [88].

A large number of transthyretin variants have been identified in the amyloid fibrils or plasma of patients with hereditary amyloidosis. These are listed by Merrill Benson in *The Metabolic and Molecular Bases of Inherited Disease, 2001* [88]. While the distribution of the amino acid substitutions gives no clue to the mechanism of fibrillogenesis, all except one involve single amino acid substitutions that result from single nucleotide mu-

tations in coding regions. The substitutions range in location from amino acid residue 10 (Cys10Arg) to residue 122 (Val122Ile) with 15 mutations in exon 2, 19 in exon 3, and 7 in exon 4. These findings provide a basis for classifying the transthyretin amyloidoses.

The most predominant variant found in kindreds of various ethnic origins with familial amyloidosis is Val30Met. Perhaps the abundance of this variant is largely due to the emergence from one founder, supposedly a Portuguese sailor. Yoshiyuki Sakaki in 1989 [108a], using polymorphisms within the transthyretin locus, found three distinct haplotypes in several Japanese kindreds with familial amyloidosis. Two recombinant events would be required for the haplotypes to originate from one founder. The probability for this recombination is lower than the mutation rate, suggesting that the mutations originated from independent origins. Another suggestion was that the frequency and diverse ethnic distribution of the Val30Met variant is due to mutational 'hot spots' composed of a CpG dinucleotide. The cytosine in a CpG dinucleotide is often methylated, and methylated cytosines can be deaminated and converted to thymidines. Routine serum electrophoresis does not identify variant forms of transthyretin but both two-dimensional polyacrylamide gel electrophoresis and a system called 'hybrid isoelectric focussing' have successfully discriminated several variants from normal transthyretin [109].

Detection of gene carriers

Identification of carriers of genes associated with amyloidosis is important for individuals who have the clinical disease and those who have not yet developed evidence of amyloidosis. In the former, identification of a mutation in a gene coding for one of the amyloid-associated proteins such as transthyretin usually allows a proper diagnosis. For individuals in families with hereditary amyloidosis, detection of disease-associated genes is of value for genetic counseling. The autosomal dominant amyloidoses are late-onset diseases, so gene carriers usually have children before disease onset. Since the autosomal dominant inherited amyloidoses are associated with variant forms of plasma proteins, it is possible to detect gene carriers by isolating and analyzing the plasma protein in question, for example transthyretin. DNA analysis has been used extensively for detecting variant amyloid-associated genes. Yoshiyuki Sasaki's group [95] developed the first DNA test for the transthyretin methionine 30 gene using a specific restriction endonuclease and Southern blot analysis. The methionine 30 mutation creates a recognition site for *Ns*iI and *Bal*I, thus giving new hybridization bands on Southern blot analysis [110]. Similarly, the transthyretin alanine 60 variant gene can be detected using *Pvu*II since the A → G change in codon 60 creates a new *Pvu*II site [98]. Southern blot analysis, however, usually requires the use of radioactive probes and relatively lengthy hybridization schedules followed by exposure of radiographic film. These methods were superceded by PCR techniques combined with restriction endonucleases. The transthyretin gene exons have the advantage of being small and easily amplified by PCR [111]. These methods can be used on tissues and even on histology sections of organs from individuals who have died. They can also be applied to amniotic fluid cells or chorionic villus biopsy for prenatal diagnosis [112, 113].

Animal transthyretin and the transgenic mouse

The only animals that have a hereditary form of amyloidosis are early senescence mice that have a mutation in the apolipoprotein AII gene [114, 115]. Even though mice have transthyretin, murine transthyretin-associated amyloidosis has not been reported. A considerable degree of homology was found between the amino acid sequences of mouse and human transthyretin [103]. Among the 127 amino acids of the mature human transthyretin, 25 are replaced by different amino acids in the mouse transthyretin. Interestingly, 24 out of the 25 substitutions are located on the outer surface of the protein. Thus, the regions corresponding to functional domains are highly conserved between mouse and human. In addition, the sequences of the 5′-flanking region are highly conserved and the homology from the putative TATA box to the –189 region is 85%. These results suggested that the human transthyretin gene could be transcribed to produce protein that is functional in the mouse. With the advances in molecular technology and sequencing of the transthyretin gene, transgenic animals could be produced. It was hoped that transgenic mice carrying the human Met 30 transthyretin gene could provide a model for familial amyloidotic polyneuropathy.

In 1986, Sasaki et al. [116] introduced a mouse metallothionein-I human Met 30 transthyretin fusion gene into mouse embryos. The mouse metallothionein-I promoter induced by heavy metals was used for efficient expression of the Met 30 transthyretin protein in the liver. Variant human transthyretin was produced in these mice but at low levels. The Japanese group of Ken-ichi Yamamura [117] microinjected a 7.6-kb human Met 30 transthyretin gene containing a 500-bp upstream region into fertilized eggs of C57BL/6 mice. Val30Met transthyretin was specifically expressed, with levels increasing during early development and peaking at 17 days gestation. The level in yolk sac was higher than in the liver and was constant during development. Thus in transgenic mice, developmental regulation of the human transthyretin gene in liver and yolk sac is exactly the same as that of the endogenous mouse transthyretin gene. Transthyretin mRNA was not

detected in other tissues, including brain, although mRNA has been demonstrated in the rat choroid plexus. This result suggests that the control region of transthyretin gene expression in the liver and yolk sac is retained within the 500-bp upstream region of this gene. The regulatory element, which directs transthyretin gene expression in brain, may be in the more upstream region. Human transthyretin was found in the serum and shown by immunological tests to have at least two antigenic determinants, one of which is common to that of human transthyretin. These results suggest that human transthyretin was coupled with mouse transthyretin to form tetramers in transgenic mice.

The overall expression of transthyretin in the previous experiments was one-tenth of mouse transthyretin expression, suggesting that the 5′ regions required for efficient expression in the liver were absent. The low levels of expression also suggested that the formation of amyloid fibrils might not be possible in these mice. In a further study, Ken-ichi Yamamura's group [118] showed that distant enhancer elements 1.96 and 1.86 kb upstream increase transthyretin production in the liver ten-fold. A 6-kb fragment that contained the distal enhancer was isolated and used to produce transgenic mice. In these experiments, amyloid deposits were not found in animals aged 12 months or less, but were found in older animals aged 15–18 months.

The transgenic mouse and amyloid production

Shimada et al. [119] also used the metallothionein-I promoter region to obtain adequate expression of the Met 30 transthyretin gene in mice. Low expression was found in liver, heart, brain skeletal muscle, kidney, and lung. Deposition of amyloid began in the mucosa of the small intestine of 6-month-old mice. Amyloid deposits were also observed in the renal glomeruli of 12-month-old mice. In contrast to familial amyloidotic polyneuropathy produced in humans as a consequence of the Met 30 gene, amyloid deposits were not evident in peripheral nervous tissues or in the choroid plexus. Shukuro Araki and coworkers [120] also produced transgenic mice carrying the gene for human Val30Met transthyretin. They also found no amyloid deposition in the nervous tissues of transgenic mice up to 24 months of age. In all autopsies of human familial amyloidotic polyneuropathy patients, Araki's group [121] had observed marked amyloid deposition in the choroid plexus and substantiated the local histochemical localization of human transthyretin in the choroid plexus epithelial cells. They speculated that the production of human variant transthyretin by the choroid plexus is related to amyloid deposition in the peripheral nerve tissues. In the transgenic mice, however, they showed immunohistochemically that there is little or no production of human transthyretin in the epithelial cells of the choroid plexus and they hypothesized that this might be the reason for the lack of amyloid deposits in the peripheral and autonomic nervous system of the mice. They also suggested that since, in humans, degenerative changes in the peripheral nerve tissues were observed before amyloid deposition, there could be other intrinsic factors involved in amyloid deposition in familial amyloidotic polyneuropathy. To test this speculation in the case of the choroid plexus, Araki proposed production of a transgenic mouse that expressed mutant human transthyretin in the brain. In 1991, Kiyoshi Ohuchi and coworkers [122] carried out this experiment. A minigene in which the promoter region of the mouse myelin protein gene was ligated to human mutant transthyretin cDNA was used for microinjection. This promoter was used because it had been found to express mouse myelin basic protein cDNA efficiently and specifically in the brain of transgenic mice [123]. The construct did produce human transthyretin in cultured mouse L cells. Analysis of the resultant transgenic mice showed only human transthyretin mRNA expression in brain but at half the level of mouse transthyretin mRNA in brain. Human transthyretin protein was not detected. No sequence variation such as deletion or rearrangement was present, and the human transthyretin protein was not lost to amyloid deposition since no deposits were found. The authors suggested that because myelin basic protein is produced in oligodendrocytes, human transthyretin mRNA is probably present in the same cells of the transgenic mouse. If the translation of the human transthyretin mRNA is restricted, or human transthyretin is rapidly degraded in oligodendrocytes, the human transthyretin minigene would be better expressed in other cells of the brain, and they planned to experiment with promoter regions of other genes.

Yamamura et al. in 1986 [117] and Costa et al. in 1989 [102] had reported that transthyretin mRNA was expressed in both liver and the brain from the same RNA start site. The RNA start sites in the liver of the mouse [117], rat [104], and human [97] were reported at homologous positions, but no data had been reported on the start sites in brain. In 1989, Kiyoto Motojima and Sataro Goto [108] showed that the brain has an additional transcription start site for mRNA. This site is located at least 65 bp upstream of the previously published one, passing over a TATA-like sequence, and it is used significantly in the brain but little in the liver. Another TATA-like sequence, 5′-AATAAT-3′ was found in the upstream region. The possibility exists from their results that the brain uses both RNA start sites, thus the choroid plexus may have a different promoter from the liver.

In his 1989 experiments, Shimada had shown that the amyloid deposits in the transgenic mouse showed both human variant transthyretin and the mouse serum amyloid P component [124]. The main component of amyloid

fibrils is different and specific to each type of amyloidosis. However, a minor component common among the amyloidoses is amyloid P [125], which is derived from, and identical to, the serum amyloid P component. The cDNA and genomic clones for both human and mouse serum amyloid component had already been isolated [126–128]. Ken-ichi Yamamura and his group [128] decided to investigate the effect of human serum amyloid P component on amyloid deposition in transgenic mice expressing the Val30Met human transthyretin variant. Two lines of transgenic mice were produced, one expressing the mutant human transthyretin gene and the other the human serum amyloid P component gene. These two lines of transgenic mice were mated to produce double-transgenic mice carrying both mutant genes. The serum concentration of human serum amyloid P component in these transgenic mice was 42 µg/ml and was about equal to that in human control serum. In the double-transgenic mice, the onset, progression and tissue distribution of amyloid deposition were the same as in the single-transgenic mouse. These results suggest that serum amyloid P component is not important for the initiation and progression of amyloid deposition.

In a study by Araki and coworkers in 1991 [118], the Val30Met variant mice were further analyzed at 24 months. They then showed amyloid deposition in stomach, intestine, glomeruli, heart, vascular system, and thyroid gland, but not in brain, choroid plexus, peripheral nerves or hematopoietic tissues. The mice showed an increase in amyloid deposition with age, consistent with familial amyloidotic polyneuropathy, except for the absence of involvement of peripheral nerve, choroid plexus, and hematopoietic tissue. Deposition was prominent in tissues with a rich blood supply such as kidneys, heart, and thyroid gland but not in the organs where the precursor protein is expressed (liver and choroid plexus).

With a view to determining if endogenous mouse transthyretin was preventing amyloid deposition in the nervous system of the transgenic mouse, Shuichiro Maeda et al. [129] disrupted the mouse transthyretin gene by gene targeting. The targeting vector contained a neomycin gene that served as the marker for positive integration, along with a 5′ and 3′ sequence with homology to the mouse transthyretin locus. An HSV-tk gene was placed at the end of the region of homology to serve as a marker for negative integration. The vector was injected into cultured mouse embryonic stem cells. Homologous recombination was seen in 1 out of 80 cells that were used to produce chimeras. An unexpected result of the experiment was that homozygous transthyretin-deficient mice appeared normal. This was confirmed in a second experiment [130], in which disruption of the transthyretin gene in mouse was accomplished using the technique of gene targeting in embryonic stem (ES) cells. Since transthyretin is not expressed in ES cells, a positive-negative selection strategy was used. The targeting vector was a replacement vector containing two selectable markers, the bacterial neomycin-resistant gene for positive selection and the herpes simplex virus thymidine kinase gene for negative selection. The MC1 neomycin expression cassette was introduced into the second exon of a 5.9-kb genomic mouse transthyretin gene fragment that carries exons 1–3. The MC1 thymidine kinase cassette was added at the 3′ end of the transthyretin gene. After transfection and injection of the ES clones into MF1 host blastocytes, two germline chimeras were obtained. These animals were bred with MF1 females and transmitted the disrupted transthyretin allele to 50% of their progeny. Heterozygous animals were intercrossed and genotyping of the resulting progeny showed that, with an average litter size of eight, live-born mice homozygous for the disrupted transthyretin gene were recovered at the predicted frequency, indicating that absence of the transthyretin gene does not compromise fetal development. Further breeding experiments showed that the fertility of homozygous mice of both sexes was normal. Verification that the targeting event had in fact generated a null mutation at the transthyretin gene locus was carried out by examining of transthyretin levels in peripheral blood by Western blot analysis. Rat transthyretin-specific polyclonal antiserum that cross-reacts with mouse transthyretin was used. The rat and mouse transthyretin differ in only seven amino acid residues. The mutant animals showed total plasma thyroxine levels of about 50% compared to the wild-type mice. There was increased binding of thyroxine to thyroxine-binding globulin, while free thyroxine levels were the same as in the wild-type mice. Total and free tri-iodo thyronine and thyrotropin-stimulating hormone levels were unaffected by the mutation [131]. Because the animals were completely healthy, it was surprising to find low plasma RBP levels and plasma retinol below the level of detection. However, this did not have a visible effect on normal development or growth. Thus, a surprising result of these gene-targeting experiments was that transthyretin is evidently not essential for normal development, viability, or fertility. The finding that the development of the null transthyretin mice was normal had not been expected, since transthyretin is highly conserved and found in many species such as birds and reptiles. If transthyretin were found to be unnecessary to human development and survival, it could influence treatment of familial amyloidotic neuropathy in humans.

In 1997, collaboration between the research groups of Shuichiro Maeda, Vasso Episkopou, and Maria Saraiva resulted in a detailed analysis of the transgenic animal model (mouse) for homozygous familial amyloidotic polyneuropathy and answered the question of whether the endogenous mouse transthyretin interfered with the formation of amyloid [132]. Their experiments used two ge-

netic strains of mice, one deficient in transthyretin generated by gene targeting as described above [130] and a wild-type transgenic mouse carrying the human mutant transthyretin gene with its cognate 6-kb upstream region. The laboratory of Ken-ichi Yamamura [133] had previously found that this 6-kb upstream region is able to direct, not only the developmental and tissue-specific expression of the transthyretin gene in the transgenic mouse, but also the amount of protein expressed. The transgene, 6.0-hMet 30, was expressed in the liver, choroid plexus, kidney, and yolk sac, the same tissues where endogenous transthyretin is found. The levels of expression were also equivalent to those of the endogenous mouse. Crossing of the two mouse lines produced transgenic mice that lacked endogenous mouse transthyretin and expressed human Met 30 transthyretin. This allowed for comparison of the onset and distribution of amyloid deposition between these mice and wild-type transgenic mice expressing both endogenous mouse transthyretin and human mutant transthyretin genes. Amyloid deposits were first observed in the esophagus and stomach when the mice were 11 months of age. With advancing age, amyloid was deposited in other tissues. No significant difference was detected in the onset, progression, and tissue distribution of amyloid deposition between the two sets of mice. The authors concluded that endogenous normal mouse transthyretin probably does not affect deposition of amyloid derived from the Met 30 variant human transthyretin in these mice. Although amyloid deposition in the peripheral nerve has not yet been observed in transgenic mice, these mice have provided a means of not only defining the factors that modulate the initiation and progression of amyloid deposition, but of searching for drugs which could prevent amyloid deposition. The finding that the onset of amyloid deposition occurred in these mice as early as 6 months of age was rather surprising since disease in humans with familial amyloidotic polyneuropathy usually takes 20–45 years to develop. Thus, the transgenic mouse system shows promise of application to the development of a mouse model for other late-onset and dominantly inherited diseases such as Alzheimer's disease.

The transgenic mouse and retinol levels

The production of a transthyretin-deficient mouse line by Episkopou et al. [130] allowed this laboratory to explore not only the effect of the absence of transthyretin on the viability and development of the mice and the effect of endogenous mouse transthyretin on amyloid deposition, but also to explore the effects of transthyretin deficiency on retinol and RBP metabolism [134.] These experiments also led to very unexpected results. In pooled plasma from the transthyretin-null mice, retinol levels averaged 6% of those in the wild-type animals. Similarly, plasma RBP levels in null mice were 5% of wild-type levels. This level of plasma retinol, when seen in wild-type mice deprived of retinol, is associated with severe vitamin deficiency. Those mice would be blind, undergoing extreme weight loss, and, unless retinol or retinoic acid was restored to the diet, close to death. In fact, the null mice were found to be phenotypically normal and fertile and had the same longevity as wild-type mice. Hepatic retinol and retinyl ester levels were similar for mutant and wild-type mice, suggesting that the mutation does not affect either the uptake or storage of dietary retinol. Levels of retinol and retinyl esters in testis, kidney, spleen, and eyecups from the mutant mice were all found to be normal. In addition, levels of cellular RBP, type I, which are regulated by nutritional retinol status, were equivalent in the mutant and normal mice. The authors concluded from these data that retinol-dependent responses and functions are not impaired in the mutant mice, and that they can take up dietary retinoid and also transport retinol to their tissues at rates sufficient to maintain normal vitamin A status. Previously (see discussion above), the predominant mechanism for delivery of retinol to the appropriate tissues had been considered to be by means of complexation with RBP. Based on these experiments, the Episkopou group asked the question 'In view of their low plasma retinol-binding protein levels, how do the null mice receive sufficient retinol?' They suggested that one possibility, based on the findings of the laboratory of Scarlata [135], is that the tissue uptake of plasma retinol is more efficient when the retinol/RBP complex is not complexed with transthyretin. The Scarlata laboratory had shown that the rate of dissociation of retinol from the RBP/transthyretin complex is 2.5-fold slower than its dissociation from RBP alone and suggested that transthyretin plays a role in modulating the release of retinol from RBP. Thus, the absence of transthyretin in the null mice may facilitate retinol uptake by tissues and this may account for the lack of retinol deficiency in the tissues of the null mice. This hypothesis is further supported by the observation from the laboratory of Zech [136] that for adult male rats with low vitamin A status and reduced plasma retinol levels (averaging 2–7 μg of retinol/dl), the daily utilization rate of retinol is less than 10% of the retinol moving through the plasma. Therefore, in these rats, the amount of circulating retinol is still in excess of the amount actually used by the tissues. A second hypothesis of the Episkopou group takes into account the fact that although plasma retinol levels in the transthyretin-null mice are low (1–2 μg of retinol/dl), comparable to those observed in the later stages of vitamin A deficiency, unlike vitamin A deficiency, the supply of retinol to tissues in the transthyretin-null mice is constant. Thus, the lack of transthyretin possibly helps to make the tissues of the mutant mice retinol sufficient. The null

mice may have reduced the needs of their tissues for retinoid by lowering the rate of tissue retinoid catabolism. At present there are no data to support this hypothesis. A third explanation may be that the survival of the null mice indicates that tissue retinoid needs can be met by an alternative retinoid delivery system. Plasma all-trans-retinoic acid levels in the mutant mice were 2.3-fold higher than those of wild-type mice. Plasma and tissue retinoic acid levels may be elevated in the null mice to compensate for the relative absence of retinol in the circulation. This may reflect a physiological adaptation that permits the mutant mice to utilize efficiently this retinoid delivery pathway. A fourth possibility is that retinyl esters, transported from the gut to the liver in chylomicrons, may supply retinol to the tissues. William Blaner et al. [137] had previously described this process for delivery of retinyl esters to adipose tissue.

In addition, data on the transthyretin-null mice do not unequivocally support or disprove the hypothesis that transthyretin prevents the filtration of RBP from the circulation although kidney RBP levels were similar for the mutant and wild-type animals and the authors were unable to detect intact RBP in the urine from the null mice. The authors conclude that there is a partial blockage in the secretion of RBP from the hepatocytes of the mutant mice that leads to decreased plasma levels of the complex of RBP with retinol rather than increased loss through the kidney. Further investigation is necessary to determine which hypothesis for the sufficiency of tissue retinol in the null mice is correct.

Original studies had proposed that the transthyretin/RBP complex was formed in the plasma after both proteins were independently secreted from cells. More recent studies including those described above on the transthyretin-null mouse by Wei et al. [134] and the results from the laboratory of Rodolfo Berni, which showed that retinoids interfere with the recognition between RBP and transthyretin and thus inhibit RBP secretion, suggest that the transthyretin RBP complex may be formed within the hepatocyte [71, 138]. Using HepG2 cells as a model system, the laboratory of Sancia Gaetani [139] showed that association of the two proteins occurs within the cell. The intracellular complex was detected only when metabolically labeled cells were lysed under mild detergent conditions, followed by immunoprecipitation and SDS-PAGE. In addition, cells treated with an agent to block the exit of proteins from the endoplasmic reticulum showed that the transthyretin/RBP complex was present in the microsomal fraction. The authors concluded that the complex is formed within the cell and, in particular, within the endoplasmic reticulum. Since neither protein has an endoplasmic reticulum retention signal, the authors concluded that chaperones might possibly play a part in the retention of transthyretin and RBP in the endoplasmic reticulum. They found that calnexin, an endoplasmic reticulum integral membrane protein which functions as a chaperone, co-precipitates with RBP and transthyretin when cell lysis and immunoprecipitation were performed under mild conditions. They suggest that calnexin may be involved in RBP and transthyretin retention in the endoplasmic reticulum and in transthyretin tetramer formation and possibly formation of the complex of transthyretin and RBP.

In 1997, the laboratory of Shigeaki Kato investigated the interaction of transthyretin and RBP in the uptake of retinol by primary rat hepatocytes [76]. Using tritiated retinol, they followed the uptake through the transthyretin/RBP complex in parenchymal and non-parenchymal cells. The cellular accumulation of retinol was time and temperature dependent in both types of cell. In parenchymal cells, the incorporated labeled retinol was converted to retinyl ester; in non-parenchymal cells, it remained mainly as unesterified retinol. The amount of retinol taken up from the transthyretin/RBP complex was nearly twofold greater than that from RBP alone. An excess of either retinol/RBP or retinol/RBP/transthyretin complex inhibited the uptake of labeled retinol from protein-bound retinol. Furthermore, an excess of free transthyretin inhibited retinol uptake through the RBP/transthyretin complex. The authors postulated that transthyretin may participate as a positive regulator in the delivery of retinol bound to RBP from plasma, possibly by a membrane receptor, and that retinol uptake takes place preferentially from the transthyretin/RBP complex into both parenchymal and non-parenchymal cells. The uptake of labeled retinol by parenchymal cells was saturated, whereas uptake by non-parenchymal cells was not. They suggest that the physiological importance of transthyretin in retinol delivery may be especially important to vitamin-A-storing stellite cells in the non-parenchymal fraction. The data thus far, however, suggest that our present understanding of the retinoid delivery and utilization system based on the role of RBP must be revised.

Structure of the amyloid fibril with transthyretin

In parallel with the progress made in the molecular biology of the transthyretin gene, the ability to carry out prenatal testing for abnormal genes, the production and structural studies on the variant homozygous transthyretin proteins, and the development of an animal model for familial amyloidotic polyneuropathy, studies were also on-going into the structure of the amyloid fibrils and the mechanism of fibrillogenesis. The X-ray structures of the variant transthyretin proteins, while showing changes in tertiary conformation due to the point mutations that could be interpreted in terms of possible amyloid fibril structures, yielded no overall unifying conformational change that could result from all the various point mutations known to result in amyloidosis.

While each variant might, in fact, form a fibril by a different mechanism due to conformational changes specific to a particular point mutation, a general mechanism of fibrillogenesis, at least for a particular precursor protein, seems more satisfying, especially considering the a large number of variants is now known in the case of transthyretin, all of which cause amyloid deposits. An additional complication to the picture was the finding that the fibrils in senile systemic amyoidosis, affecting about 20% of the population over 90 years of age, consisted of normal transthyretin [140–142].

A recent publication by a Swedish group [143] compared all 23 of the presently available X-ray structural studies on transthyretin variants. These structures were compared to a new higher-resolution native structure (1.5 Å). The authors concluded that the previously reported structural differences are not significant. In addition, they compared their native transthyretin structure, whose crystals were grown at physiological pH in low salt and polyethylene glycol, with transthyretin models determined at pH 5.0–5.5. On the basis of examination of residues which could change their charge with the change in pH, they concluded that the wild-type structure and probably also structures of the variants are identical at pH 5.3 and pH 7.0 (at least in the presence of high salt).

Early studies on the fibrils had involved attempts to solubilize them and determine their protein composition [54]. This study showed that the fibrils contained the variant protein, in this case the Met 30 variant, but some doubt remained as to whether the variant protein was in the form of a tetramer, dimer, or monomer. Since the vast majority of individuals with familial amyloidotic polyneuropathy are heterozygous for the transthyretin variant, a statistical distribution of wild-type and variant subunits would be expected in tetrameric transthyretin in human plasma. Usually in these individuals, the plasma transthyretin is depleted to some extent of variant subunits and the fibrils are composed predominantly of variant transthyretin [144]. This led to the theory that the variant protein may have a different conformation from the wild type and that this conformational change made the variant amyloidogenic. This result stimulated a series of X-ray structural studies on the variant protein.

A subsequent series of studies on amyloid fibrils showed that they were composed of both full-length transthyretin and fragments of the molecule [92, 99, 145, 146]. N-terminal microheterogeneity was found in the transthyretin isolated from amyloid fibrils by these groups. The reason for this was presumed to be enzymatic cleavage, possibly after formation of the fibrils. In the studies of Dwulet and Benson, the transthyretin isolated from the fibrils contained a point mutation, Met 30, as well as Val 30, indicating coexpression of two transthyretin genes. The other studies found only transthyretin with the Val30Met point mutation. This same mutation was present even though the individuals with familial amyloidotic polyneuropathy were Portuguese [146] or Japanese [145, 146]. To explain the finding of both normal and variant transthyretin in the fibrils, Dwulet and Benson proposed that the fibrillogenic mechanism probably involved the deposition of either transthyretin dimers or tetramers. In 1987, the research group of Per Westermark analysed amyloid fibrils from a Swedish population [147, 148] and also found N-terminal microheterogeneity. In addition, they found that most of the transthyretin-like protein was covalently bound as dimers, heterodimers, or polymers [149]. The covalent link was hypothesized to be a disulfide bond, formed from the cysteine residues at position 10 in the transthyretin molecule. Since these cysteine residues are not close together on the transthyretin tetramer, they postulated that fibrillogenesis takes place using the transthyretin monomer or involves abnormal transthyretin dimers. It was not clear at this point whether the formation of disulfide bridges represented a post-fibril formation process or whether it was of importance in fibrillogenesis. In 1993, the research group of Colin Blake proposed a model for the amyloid fibril that involved polymerization of near-native transthyretin tetramers via disulfide bridges [150]. The authors proposed that the effect of the substitution of methionine for valine at position 30 is transmitted through the protein core to Cys10, which, as a result, becomes slightly more exposed. Their results on the X-ray structure determination of a crystal of homozygous recombinant Val30Met transthyretin showed no other major differences in conformation from that of the wild-type structure. These findings contradicted a study by Hamilton et al. [49] of the X-ray crystal structure of naturally occurring Met 30 transthyretin isolated from the plasma of an individual who was homozygous for the variant. In this structure, the internally substituted Met 30 caused the outer sheet of the β barrel in the region of the substitution to be slightly forced apart. This then resulted in an increase in the size of the thyroxine-binding cavity, agreeing with experimental data showing that the affinity of the Met 30 for thyroxine was decreased. These differences in structure between the naturally occurring variant protein and the recombinant have not yet been resolved or explained.

Colin Blake used computer graphics to show that it was possible to model a linear aggregate of transthyretin molecules, each linked to the next by a pair of disulfide bonds involving the lone cysteine residue at position 10. Formation of these disulfide bonds involves a small number of slightly short molecular contacts with native transthyretin molecules, most of which are relieved in the Met 30 variant. However, opposing this idea is the fact that there is one transthyretin mutation associated with familial amyloidosis resulting in a substitution of Cys 10 with Arg, making disulfide bond formation impossible [151]. However, this does not completely rule out a pos-

sible role for disulfide bridge formation in fibrillogenesis since, if the transthyretin tetramer is the fibril building block, in heterozygous individuals both variant and wild-type protein may be present in the tetramer and the variant protein may induce fibrillogenesis while the wild-type stabilizes the fibrils via disulfide bridges.

Prior to the experiments of the Westermark group, current theory of fibrillogenesis still involved the requirement for a variant transthyretin, usually a point mutation, which was believed to be amyloidogenic, i.e., prone to form amyloid fibrils. Work with amyloid fibrils common in age-related systemic amyloidosis showed that there is no abnormality in the primary structure of transthyretin in this disease and that, in addition to full-length transthyretin protein, the fibrils contain transthyretin fragments lacking a significant part of the N-terminal end [142, 143, 148]. Furthermore a predominance of the transthyretin fragments contained residues 76–82. The question was posed 'Is this due to a selective or random process?' Despite a considerable body of research, this question has still not been answered satisfactorily. Senile systemic amyloidosis is a disease of advanced age and few affected individuals are below 80 years old. Although the disease is systemic, the deposits are generally small and mainly affect vessels in different parts of the body. Therefore, it is usually a benign disorder but in some individuals, mainly men, it gives rise to severe cardiomyopathy, often with ultimate cardiac insufficiency [149]. All sequence studies on the protein [152], cDNA [153] and gDNA [154] levels have shown that no amino acid substitution exists in this form of the disease. Instead, in addition to an instrinsic amyloidogenic property of wild-type transthyretin, some yet unknown age-associated factor is involved in the pathogenesis. A recent study by Kishikawa et al. [155] has shown by mass spectrometry that S-sulfonated transthyretin is present in individuals with senile amyloidosis. When reduced with dithiothreitol, this transthyretin did not show Congo red binding or turbidity indicative of fibril formation. They suggest, on the basis of these results, that S-sulfonated wild-type transthyretin is highly amyloidogenic and that exogenous sulfite may be a cause of senile systemic amyloidosis [156]. Another study by Suhr et al. [157] investigated the thiol conjugation of transthyretin and its relation to age and symptomatic amyloid disease. Their results showed that thiol conjugation is dependent on age and the presence of symptomatic amyloid disease and varies between different populations. They also showed that variant transthyretin is more susceptible to thiol conjugation than the wild-type protein. They suggest that post-translational factors may be related to amyloid formation.

Production of synthetic amyloid fibrils from transthyretin

In addition to examining the material involved in the amyloid fibril, experiments began which aimed at making synthetic fibrils to be compared with those formed in vivo, and structural studies were initiated on both the synthetic and natural fibers. In 1991, the laboratory of Per Westermark [158] showed the formation of fibrils from normal transthyretin and synthetic transthyretin fragments, preferentially those corresponding to the β strands. They found that a number of fibrils were formed when normal transthyretin was dissolved in acetic acid. These were about 10 nm wide, non-branching, and 'slightly wavy.' The synthetic transthyretin fragment with residues 10–20 gave rise to a large number of fibrils in acidic pH, while the fragment with residues 105–115 first formed fibrils at neutral pH. The fibrils were of varying length, non-branching, about 10 nm in width and of 'amyloid-like appearance.' The fragment with residues 50–60 was not fibrillogenic. The synthetic fibrils from the transthyretin fragments stained with Congo red, as do those of in vivo amyloid. The authors suggested that their findings were consistent with the β strands A and G being strongly fibrillogenic. They also found that peptides corresponding to the DE loop and the lone α helix both readily formed amyloid-like fibrils in vitro. When the helix was included in a longer peptide with the flanking regions, no fibrillogenesis was seen [159]. They also questioned the then current theory of a point mutation in the transthyretin as a prerequisite to fibrillogenesis.

This work emphasized the presence of truncated forms of transthyretin as well as the full-length protein. Pettersson et al. in 1987 [147] showed that transthyretin is found with slightly truncated N-terminal ends in normal plasma. The studies of the Westermark group had identified the positions of the splitting in the native protein recovered from amyloid fragments in individuals with senile age-related amyloidosis and had suggested it was not random but occurred regularly after residues 45, 48, and 51, i.e., in the C strand or the CD loop region [153, 156]. Some other minor cleavage points were also found. Similar cleavage had been found in the variant protein recovered from the amyloid fibrils found in individuals with familial amyloidotic polyneuropathy [160, 161]. Whether the cleavage is a post-fibrillogenic event or is of importance in the process of fibrillogenesis is unclear at the present time. The research group of Jeffery Kelly [162] has proposed that the C and D strands are weakly interacting with the rest of the transthyretin β sandwich and that therefore an amyloidogenic folding intermediate could develop in which the C and D strands make a loop that is displaced from the rest of the molecule. According to this hypothesis, and as indicated by the fact that the transthyretin monomer is stable, the cleavage sites found

in the transthyretin monomer are a post-fibril event. Some findings are, however, difficult to correlate with this hypothesis. First, in several amyloid fibrils, full-length transthyretin makes up a minor portion of the fibril protein, perhaps not more than 10–20% of amyloid transthyretin. Furthermore in sequence analysis of truncated proteins, the research group of Westermark was only able to identify C-terminal fragments starting at positions 46, 49, 52, and 53 [156]. An N-terminal fragment has not been identified.

In another study by the Westermark group, various antigenic mapping procedures were used to determine whether major antigenic sites differ between normal transthyretin, transthyretin obtained from amyloid fibrils, and in situ amyloid fibrils [163]. The results showed that the antigenic sites on normal plasma transthyretin include the AB loop and the CD loop that are on the outside. Antiserum against β strand H reacted only with transthyretin in amyloid fibrils and transthyretin isolated from amyloid fibrils but not with normal plasma transthyretin. These results suggest that the conformation of transthyretin within the amyloid fibrils is altered compared with plasma transthyretin. These changes occur in the partially fragmented transthyretin and also in the H strand, which is important since this strand participates in formation of the transthyretin dimer. In addition, the use of different antibodies in an ELISA system indicated that C-terminal fragments of transthyretin were present over the whole range of peptide sizes whereas there was a big peak corresponding to full-length transthyretin monomer. Only a very small peak with immunoreactivity and the size of a small N-terminal fragment was found [154], which the authors conclude reinforces the possibility that cleavage occurs before fibrillogenesis and is important in amyloidogenesis in at least some amyloid syndromes. There is some evidence from a different source to support the idea that the proteolysis observed in these studies may not occur before deposition in amyloid but afterwards. Experience with the treatment of familial amyloidodotic polyneuropathy by means of liver transplants [164–168] has shown that the extent of transthyretin amyloid may regress (presumably involving a proteolytic process), with partial return of organ function, following transplantation.

Another fundamental unsolved question is why amyloid deposition occurs at specific locations, especially with respect to the various transthyretin syndromes. Why does one variant form deposits in nerves, another give rise to cardiac deposits, and yet another to deposits in the vitreous humor of the eye? One possibility is that the different mutations and the amyloidogenic wild-type transthyretin lead not to one but several different partly unfolded forms (normal or abnormal) with amyloidogenic properties, and perhaps by several different mechanisms. These forms may behave differently and react differently with other components such as amyloid P, giving rise to different clinical manifestations. Cleavage of transthyretin may also result in amyloidogenic fragments that differ in behavior from amyloidogenic full-length transthyretin. An interesting finding of the Westermark group is that on examination of the amyloid from the hearts of eight individuals with Swedish Met 30 amyloidosis, the cardiac tissue deposition pattern in those individuals with full-length amyloid transthyretin differs from those in which the amyloid predominantly contains transthyretin fragments [140]. The authors propose that there may be more than one pathway for amyloidogenesis, one for full-length transthyretin and one for the fragments. The latter is likely to predominate in senile systemic amyloidosis. All the evidence strongly suggests that there is a cause-and-effect relationship between specific single-site variants of transthyretin and the pathology of familial amyloidotic polyneuropathy. However, even if wild-type and variant transthyretin are both capable of being transformed into amyloid in vivo, a single pathological mechanism is still possible which could be facilitated in the case of specific variants. One would assume, since transthyretin levels are probably the same in the circulating plasma in all organs, that the protein would be available for deposition equally in these organs except, perhaps, in the brain. The observed site specificity could be due to the specific transthyretin variant involved in the amyloid deposit or to tissue-specific factors that act on the precursor protein when it achieves adequate concentration levels. In this regard, it should be noted that common tissue components have been identified in almost all amyloid deposits examined thus far.

Theories of fibrillogenesis

The research group of Jeffery Kelly undertook studies to investigate the hypothesis that the variant transthyretin proteins produce amyloid not by markedly affecting the structure of the folded state but rather by affecting the denaturation pathway and or the degradation pathway for protein turnover. Thus far they have not been able to produce amyloid in vitro under conditions where the normal tetramer is known to exist. They have concluded from this observation that amyloid is probably not derived from the tetrameric form of transthyretin [151, 169]. The group did succeed in converting wild-type transthyretin into amyloid fibrils using partial acid denaturation [169, 170]. They concluded from the fact that amyloid fibrils can be produced from transthyretin either by acid denaturation or by refolding denatured transthyretin that the amyloidogenic intermediate, i.e., the precursor that leads to amyloid fibril formation, is a normal denaturation/folding intermediate. Aggregation can occur during protein refolding after treatment with denaturants or during protein denaturation. Also well-established is that intermediates on the protein-folding pathway

can self-assemble, leading to aggregation rather than folding intramolecularly into the native non-pathogenic tertiary structure [171]. Earlier studies by Alan Cohen and colleagues had shown that amyloid fibrils are present proximal to and inside lysozomes of reticuloendothelial cells in animals with amyloid disease [172–174]. In the case of transthyretin-based amyloid diseases, fibril formation seems less likely to involve lysozomes, since appreciable amounts of proteolysis of the transthyretin are not observed in all affected individuals. However, Kelly and Lansbury [175] suggested that the lysozome could produce the environment for acid-mediated denaturation of transthyretin, resulting in a partially denatured intermediate, which could associate to form amyloid fibrils faster than it could be degraded by lysozomal proteolysis. This correlates with the results of Per Westermark who used an acidic medium to produce in vitro amyloid fibrils and also observed variable amounts of proteolysis in the transthyretin deposited in fibrils in vivo.

Kelly further supported his hypothesis with the fact that amyloid fibrils, once formed, are protease resistant. However, this conclusion is contrary to the behavior of amyloid deposits in situ which, as discussed above, can regress when the precursor pool from which the deposits are drawn is depleted. Building on the research of Tandon and Horowitz [176, 177], Kelly constructed a system for examining the denaturation process of transthyretin without competition from the process of aggregation. This system depended on the use of the zwitterionic detergent Z 3-14 which, according to Tandon and Horowitz, forms micelles which probably mimic the function of molecular chaperones during protein refolding. Kelly hypothesized that the detergent would bind to the amyloidogenic intermediate and selectively prevent self-assembly. Kelly described the quaternary structure of intermediates present under acidic conditions where fibril formation occurs in the absence of detergent. The hypothesis is that the detergent, in binding to the amyloidogenic intermediate in a reversible fashion, allows the transthyretin to undergo further conformational changes without self-assembly. Kelly proposed the following pathway for denaturation and fibril formation. Isolated wild-type transthyretin remains stable and in the tetrameric form from pH 7 to 5 and does not form amyloid fibrils. However, over the pH range 5.0–3.9, the transthyretin tetramer dissociates to monomers, which exhibit an altered but defined tertiary conformation, as probed by fluorescence and far and near UV circular dichroism. The extent of observed amyloidogenesis correlates with the concentration of amyloidogenic monomer, which is maximal at pH 4.4 [178, 179]. After further acidification (<pH 3.9), the structurally defined monomers adopt alternative conformations analogous to a molten globule-like acid-denatured state, which forms low-molecular-weight aggregates but not amyloid fibrils [180]. Kelly hypothesized that amyloidogenesis results from interactions between specific structural elements of alternatively folded transthyretin monomers. Using deuterium-proton exchange monitored by two-dimensional nuclear magnetic resonance spectroscopy, the research group of David Wemmer [181] mapped out the regions in wild-type transthyretin that are destabilized under amyloidogenic conditions, at pH 5.75 (non-amyloidogenic) and pH 4.5 (amyloidogenic monomer). The differences in the backbone amide D-H exchange rates under these two conditions revealed that there is a stable core consisting of β strands A, B, E, F and the loop between strands A and B. One side of the β sheet sandwich (strands C, B, E, and F) is destabilized under conditions favoring the formation of the amyloidogenic intermediate. The authors conclude that this region of the transthyretin is destabilized both by the effects of pH changes and also by point mutations, and loses the intramolecular contacts with the core β sheet present in the native state. It is worth noting that the CBEF β sheet contains most of the point mutations associated with familial amyloidotic polyneuropathy. Goldsteins et al. [182] also suggested a model for aggregation based on destabilization of the protein by means of modification of the C and D strands. Their hypothesis was based on biochemical studies on two highly amyloidogenic recombinant mutants lacking the native D β strand.

In a collaboration between a group in Oxford University and one at the Skaggs Institute for Chemical Biology, Nettleton et al. [183] studied protein subunit interactions and the structural integrity of amyloidogenic transthyretins. They used mass spectrometry to monitor the proportion of monomer and tetramer in wild-type and variant transthyretins and found a strong correlation between the instability of the tetramer in the gas phase and the amyloidogenicity of the transthyretin variant. The solution structure of monomeric transthyretin studied using hydrogen exchange monitored by mass spectrometry showed that Val30Met transthyretin exhibits loss of hydrogen exchange protection much more rapidly than native transthyretin, suggesting partial unfolding of the β sheet structure. The authors suggest on the basis of their results that transient unfolding of the transthyretin monomer may be a possible route to fibril formation. However, a recent study by Shnyrov et al [183a] to compare the thermal unfolding of the wild-type protein, amylodogenic variants Val30Met and Leu55Pro, and a non-amylodogenic variant Thr119Met using high-sensitivity differential scanning calorimetry indicated an order of stability of wild type > Thr119Met > Leu55Pro > Val30SMet, which did not correlate with the known amyloidogenic capability of the variants.

Ferrao-Gonzales et al. [184] used hydrostatic pressure and fluorescence spectroscopy to study the pre-aggregated state of amyloidogenic transthyretin. They found that native transthyretin tetramers were denatured by high

pressure into a conformation that exposes tryptophan residues to the aqueous environment. Lowering the temperature facilitated the pressure-induced denaturation, suggesting the importance of entropy in stabilizing the native protein. The authors suggest that the altered state may be an intermediate in the aggregation pathway.

Goldsteins et al. [185] identified two cryptic epitopes in ex vivo amyloid, obtained by mild extraction procedures from the vitreous body of the eye in individuals with familial amyloidodotic polyneuropathy. These epitopes were not detected in wild-type transthyretin or two common variants, Val30Met and Leu55Pro. However, after denaturation, all transthyretin species expose these cryptic epitopes. The authors propose that the cryptic epitopes are markers for an amyloidogenic fold and have shown that their expression correlates with increased aggregation rate at low pH. They were mapped to a domain on transthyretin where most natural mutations occur and which the authors propose is displaced in the initial phase of amyloid formation, opening up new surfaces necessary for auto-aggregation of transthyretin monomers.

Sebastiao et al. [186] determined the crystal structure of the amyloidogenic Leu55Pro transthyretin. This variant crystallizes in a different space group (C2) from the wild-type protein and other variants. The observed packing contacts are considerably different from the other crystal structures; in particular, the proline for leucine substitution disrupts the hydrogen bonds between β strands D and A and results in different intermolecular contacts. On the basis of the packing, the authors propose a model for the amyloid fibril, which consists of a tubular structure with an inner and outer diameter of approximately 30 and 100 Å, respectively and four monomers per cross-section.

The structure of a highly amyloidogenic transthyretin triple mutant Gly53Ser/Glu54Asp/Leu55Ser was determined by Eneqvist et al. [187] to 2.3 Å resolution. The authors identified in the structure a novel conformation: the β slip. A three-residue shift in β strand D places Leu 58 at the position normally occupied by Leu 55, now mutated to serine. The β slip was defined best in two of the four monomers of the transthyretin tetramer and results in new protein-protein interactions in an area of the transthyretin normally involved in complexation with the RBP charged with retinol. The authors also propose a model for the amyloid fibril based on the idea that these novel protein-protein interactions in the crystal will carry over into fibrillogenesis.

The conclusions regarding fibril formation at this point suggest that, regardless of the polymerization unit, the in vitro assembly follows kinetics that are consistent with a nucleation process and accumulation progresses along a sigmoidal time course. This is consistent with in vivo studies that monitored the accumulation in mouse spleen of a different type of amyloid (AA amyloid) in the presence and absence of an amyloid-enhancing factor [188]. The pattern of amyloid distribution, however, seems consistent with nucleation events being controlled by the intervention of some tissue-specific factors.

Polymer protein unit

Much research effort has been devoted to the question of the basic unit of the precursor protein that is involved in polymer formation and hence amyloid production. The group of Merrill Benson [189] proposed that proteolytic cleavage was the initial step in fibrillogenesis. They supported the ideas discussed above that N-terminal fragments are important and that the truncated dimer, which can no longer take part in tetramer formation, was the polymer unit. A collaborative study [190] used high-resolution electron microscopy and immunolabeling to examine amyloid fibrils isolated from vitreous humor from individuals with familial amyloidotic polyneuropathy. They concluded from their results that the unit structure of the polymer was similar to the transthyretin monomer. It seemed to many of us in the field that the rather weak contacts between dimers compared to the very tight multiple contacts between monomers at the dimer interface suggested that the dimer unit was the most probable unit of the polymer. However, recently, Quintas et al. [191] showed that upon dilution, tetrameric transthyretin dissociates into monomeric species at pH 7.0 and very nearly physiological ionic strengths. The amyloidogenic variants Val30Met and Leu55Pro showed a complex equilibrium between monomers, tetramers, and high-molecular-weight aggregates. These aggregates dissociate directly to monomers on dilution. The authors suggest that the mutations in the amyloidogenic variants could perturb the structure and/or the stability of the monomeric species leading initially to the formation of soluble aggregates and, at a later stage, to insoluble amyloid fibrils. The process is apparently irreversible, and the monomeric species formed are non-native, as shown by intrinsic fluorescence and fluorescence-quenching experiments. This monomeric species does not appear to behave like a molten globule. In contrast, under similar conditions, wild-type transthyretin also dissociates into monomers but shows no sign of aggregates. These results suggest that, in contrast to the hypothesis of Kelly discussed above, low pH is not necessary to induce partial unfolding of the transthyretin. Quintas et al. [192] also observed that the tendency for aggregate formation among the variants studied correlated with their amyloidogenic potential.

Fibril models

Amyloid is a term used to describe deposits found in tissues and organs. The deposition is now realized to be a

disorder of protein metabolism, acquired or inherited, in which normally soluble proteins and polypeptides are deposited in a specific fibrous form in the extracellular spaces. At least 20 different proteins, including transthyretin, have been identified which can form amyloid fibrils and the diseases associated with these proteins vary from neurological problems such as Alzheimer's disease through type II diabetes to systemic polyneuropathies. The nature of each amyloidosis is dependent on the particular protein involved. Recently, several review articles have been published on amyloid fibrils [193, 194] and a comprehensive review on the process of development of the fibrous state from soluble protein [195].

All amyloid deposits, regardless of the precursor protein, share certain features. They all bind the dye Congo red and interact with it in such a way as to generate a characteristic and diagnostic green birefringence [85]. Under the electron microscope, the amyloid deposits are composed of uniform, straight, unbranched fibers, approximately 100 Å in diameter and of indefinite length [196]. X-ray diffraction patterns of amyloid show that the ordered, repeating, molecular structure of the fibrils consists of polypeptide chains in an extended β conformation, hydrogen-bonded into sheets which run parallel to the axis of the fibril and which have their constituent β strands arranged perpendicular to this axis, the so-called 'cross-β' conformation [86]. These observations led to the hypothesis that amyloid is a particular type of molecular structure which can be adopted by the polypeptide chains with differing primary sequence. One particular experiment of importance was the study conducted by Inouye et al. [190], which showed that the fibrils produced by transthyretin in the vitreous fluid of patients, where the Pras extraction method was not necessary, were the same as those extracted from the kidney using the Pras extraction. This was the first time the conclusion could be drawn that the resulting fibrils were not affected by the extraction method. In general, however, most of the structural studies on fibrils have been carried out on synthetic material and not on fibrils isolated from amyloid deposits.

The fibrillogenic precursor proteins are not the only components of amyloid deposits. There are non-fibrillar components which include serum amyloid P, heparin sulfate proteoglycans, and apolipoprotein E. The major focus of research has been the fibrillogenic proteins. Based on the synchrotron X-ray diffraction patterns of the Met 30 transthyretin amyloid fibrils isolated from the vitreous humor of the eye mentioned above, Blake and Serpell [197] proposed a β sheet helix model for the core of the transthyretin amyloid fibril. β-Sheets run parallel to the axis of the protofilament, with their component β strands perpendicular to the fibril axis. The β sheets twist around a common helical axis, which coincides with the axis of the protofilament, giving a helical repeat of 115.5 Å and containing 24 β strands. Essentially, there are two coaxial cylinders of radii 6.5 and 16.5 Å. Blake proposes this as a generic model for the amyloid fibril.

Structural studies by Schormann et al. [198] on an amyloidogenic immunoglobulin light-chain revealed a crystal packing of the light chain dimers which produces an overall helical spiral with pseudo-hexagonal symmetry. The unit of polymerization is the κ light-chain dimer, which then uses a twofold rotation to link to a second dimer. Thus, there are repeated twofold axes between the monomers to give the biological dimer, and between the dimers to form the polymer. There are six light-chain dimers per turn of 360°. The diameter of the helix produced is 115 Å and the rise is 38.8 Å per turn of 180°. These dimensions also fit the information from electron microscopy studies and also provide a generic model for amyloid fibril formation. An analogous situation with transthyretin could be envisioned where the unit of polymerization is also the dimer, instead of the monomer as in the model proposed by Blake. The same helical spiral was observed in other crystal structures of amyloidogenic immunoglobulin light chains [199, 200], suggesting a stable dominant superstructure.

Inoue and Kisilevsky [201] have proposed an alternative model for the *in vivo* amyloid deposit based on experimental data from high-resolution electron microscopy and immunolabeling. Sural nerve biopsies from individuals with familial amyloidotic polyneuropathy (Met 30 transthyretin variant) as well as control tissues were used. Extracellular spaces in the vicinity of myelinated and unmyelinated peripheral nerves were found to be filled with amyloid fibrils as well as deposits of amorphous material. Their results show the fibril to be composed of a surface layer and a core. The surface layer is made up of heparan sulfate proteoglycan and is externally associated with a loose assembly of 0.5- to 1-nm-wide filaments. The core is a microfibril-like structure in which amyloid P component was enclosed in a tight helical structure of chondroitin sulfate proteoglycan. The peripheral fine filaments were shown to be transthyretin by immunogold labeling. The dimensions of the transthyretin filament suggested that its basic unit is the monomer, although other recent studies have shown that the tetramer can also provide a model which fits the fibril dimensions [201]. The amorphous deposits were a mixture of individual components of the fibril. These results suggest that the main body of the amyloid fibrils in familial amyloidotic polyneuropathy is similar to those recently described for experimental murine amyloid [202] and hemodialysis-associated β_2-microglobulin amyloid [203] as well as connective tissue microfibrils [204, 205]. The differences in the fibrils of these various types of amyloid are in the peripheral filaments which are composed of a protein specific to each type of amyloid. Indeed, from the pictures of the helical spiral produced by the immunoglobulin κ light

chains, one could well imagine it twisting around an inner core, as suggested by the results of Inoue.

Thus, although remarkable progress has been made over the last 15 years in our understanding of the biology and structural basis of amyloid deposition, there remain many conflicting pieces of evidence, as seen in the discussion of the nature of the fibril structure. We can now carry out prenatal tests for the variants of the precursor proteins and thus identify those at risk for the disease state. However, liver transplanation in the case of the transthyretin-associated amyloidoses is still the only successful treatment available. The hope is that, as more is learned about the structure of the amyloid and the mechanism of fibril formation, we will achieve the ability to resolubilize the deposits or to intervene and prevent their formation.

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