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The Ile-84 → Ser Amino Acid Substitution in Transthyretin Interferes with the Interaction with Plasma Retinol-binding Protein*

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In plasma the thyroid hormone-binding protein transthyretin (TTR) forms a tight complex with the specific retinol carrier retinol-binding protein (RBP). The Ile-84 → Ser mutation and several other point mutations in TTR are associated with familial amyloidotic polyneuropathy, which is characterized by extracellular depositions of amyloid fibrils mainly consisting of mutated TTRs. The interactions with human RBP of recombinant human normal and Ser-84 TTRs were investigated by monitoring the fluorescence anisotropy of RBP-bound retinol. A nearly negligible affinity of the recombinant Ser-84 TTR for RBP was found. This result indicates the participation of a region on the outer surface of TTR that comprises Ile-84 in the recognition of RBP. In preliminary studies the Ser-84 TTR was the only one among several amyloidogenic variant TTRs to display negligible interaction with RBP. Therefore, in general a substantially altered binding of TTR to RBP is not associated with familial amyloidotic polyneuropathy. Instead, the altered binding of Ser-84 TTR to RBP appears to be responsible for an abnormal plasma transport of RBP. The recombinant normal TTR exhibits binding properties, in its interaction with human RBP, approximately similar to those of TTR purified from human plasma. Two independent and equivalent RBP binding sites on recombinant normal TTR are characterized by a dissociation constant of about 0.4 μM.

Transthyretin (TTR)[1] is a tetrameric protein of 55 kDa, containing high affinity binding sites for thyroxine and retinol-binding protein (RBP). TTR is responsible for the transport of about 20% of the circulating thyroxine in human plasma, whereas it carries up to 80% of the thyroid hormone in the cerebrospinal fluid (1). RBP is a monomeric protein of 21 kDa that specifically transports retinol in plasma. RBP and TTR are synthesized and secreted into the circulation primarily by the hepatocytes. In plasma holoRBP circulates almost entirely bound to TTR. The association of RBP with TTR increases the stability of the retinol-RBP complex (2) and is believed to prevent filtration of the relatively small RBP through kidney glomeruli (3). ApoRBP, generated from the holoprotein upon delivery of retinol to target cells, has low affinity for TTR and can thus be selectively cleared from the circulation, in the uncomplexed form, through kidney glomeruli.

The high resolution crystal structures of both TTR (4–6) and RBP (7–10) have been described. X-ray crystallographic studies have also provided molecular details of the interactions of the two transport proteins with their small size ligands. TTR is composed of four identical subunits, of approximately 14 kDa, which are assembled around a central channel containing two thyroxine binding sites. Although the two sites are structurally identical, biochemical data have revealed a 100-fold difference in thyroxine binding affinity between these sites (11). The mechanism responsible for such a difference has not yet been elucidated. RBP is a single domain protein whose main structural feature is an antiparallel β-barrel, which accommodates the retinol molecule; the cyclohexene ring is buried deep in the barrel cavity, the isoprene chain is fully extended, and the hydroxyl end group is near the entrance of the cavity. The protein surfaces involved in the recognition between TTR and RBP have not yet been defined at the molecular level. Notably, whereas no interdependence of the binding of thyroxine and of RBP to TTR was found (12), the interactions of retinol and retinoids with RBP drastically affect RBP-TTR recognition (13–16). The latter findings strongly suggest that the region containing the loops that surround the entrance of the β-barrel in the RBP molecule is involved in the interaction with TTR (10, 16). Instead, no experimental evidence has been presented as yet to indicate which region of TTR binds RBP.

The interactions of RBP with TTR have been investigated in several laboratories. The most sensitive technique used so far to study such interactions is fluorescence polarization and anisotropy. The highly fluorescent RBP-bound retinol provides an intense signal that is suitable for fluorescence polarization and anisotropy measurements. However, conflicting data have been obtained by the use of such technique as to both the binding stoichiometry of RBP to TTR, varying from 1.5 to 4 RBP sites on the tetrameric TTR, and the dissociation constant values, varying from 0.07 to 0.8 μM (12, 14, 17–19). This variability might depend on the use of different methods to analyze binding data (12, 17, 18), as well as on distinct properties of interacting proteins obtained from different sources (17). Furthermore, in one case TTR was chemically modified to provide an extrinsic fluorescent probe that was used for fluorescence anisotropy measurements (14).

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[1] The abbreviations used are: TTR, transthyretin; RBP, retinol-binding protein.

A large number of point mutations in TTR have been described. Most of them are associated with the majority of the clinical syndromes of familial amyloidotic polyneuropathy (20). In such syndromes the formation of amyloid fibrils and their extracellular deposition in several organs (like heart and kidney) and along peripheral nerves take place. Mutated TTRs represent the major component of amyloid fibrils. Several studies have been conducted to explore the mechanism whereby single amino acid substitutions in the TTR molecule lead to the amyloid formation (6, 21–24). However, it is still unclear how altered structural characteristics of variant TTRs are responsible for the amyloid fibril formation. It has been shown previously that an amyloidogenic amino acid substitution in TTR, the Ile-84 → Ser mutation, is associated with low plasma levels of RBP (25). It has been suggested that an impaired plasma transport of RBP in carriers of the Ser-84 mutation might result from an altered interaction of the variant TTR with RBP (26). Additionally, it has been shown that the Ser-84 variant of TTR exhibits a reduced affinity for thyroxine (27–29).

In the present study, we have addressed the question of whether the Ser-84 variant of human TTR displays an altered affinity for human RBP. To this end, the interactions with RBP of normal and Ser-84 TTRs, both produced by recombinant DNA technology, have been monitored by fluorescence anisotropy of RBP-bound retinol. The results of this *in vitro* study provide additional insight into the mode of interaction between RBP and TTR. Furthermore, they appear to correlate with the abnormal metabolism of RBP observed previously in carriers of the Ile-84 → Ser mutation in TTR.

EXPERIMENTAL PROCEDURES

Materials—Human holoRBP and TTR were purified from fresh plasma as described (30, 31). As the last step of RBP purification, the holoprotein was also subjected to chromatography on a human TTR-Sepharose 4B affinity column as described (10). RBP recovered from the affinity chromatography exhibited an A_{280}/A_{330} ratio of approximately 0.9. Recombinant normal human TTR and the Ser-84 variant of human TTR were expressed and purified according to Murrell *et al.* (28). RBP and TTR were quantified by their absorbance at 280 nm, using $\epsilon_{1\%,1\,cm}$ of 18.6 and 14.3, respectively (18). L-Thyroxine was purchased from Fluka (Buchs, Switzerland) and was quantified by its absorbance at 325 nm in 10 mM NaOH, using $\epsilon_{M,1\,cm}$ of 6,180 (11).

Fluorescence Anisotropy Measurements—Measurements were carried out with a Perkin-Elmer LS-50B spectrofluorometer. The intensities of the vertical ($I_\parallel$) and horizontal ($I_\perp$) fluorescence components were recorded at an angle of 90° to the vertically polarized excitation beam. A correction factor, G, equal to $I'_\perp/I'_\parallel$, the primes indicating excitation polarized in a perpendicular direction, was used to correct for the unequal transmission of differently polarized light. Fluorescence anisotropy (A) is then defined as shown in Equation 1.

$$A = (I_\parallel - GI_\perp)/(I_\parallel + 2GI_\perp) \qquad \text{(Eq. 1)}$$

Human holoRBP was titrated by adding aliquots of concentrated solutions of either plasma or recombinant human TTR to the RBP-containing sample, in the presence of 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.4, and the increase in fluorescence anisotropy of the RBP-bound retinol upon interaction with TTR was monitored. Anisotropy values for the fluorescent RBP-bound retinol were obtained using excitation and emission wavelengths of 330 and 460 nm, respectively. The values were corrected for blank readings. The temperature was kept at 20 ± 0.5 °C by circulating water through the cell holder.

Analysis of Binding Data—Binding data were analyzed according to the method of Kopelman *et al.* (17), which is an adaptation of the more popular method of Cogan *et al.* (32) generally used for the analysis of retinoid binding to retinoid-binding proteins. Briefly, the mass low equation for the binding of RBP to TTR, shown by Equation 2,

$$K_d = nC_{TTR}\, C_{RBP}/C_{RBP\cdot TTR} \qquad \text{(Eq. 2)}$$

was used to derive a working equation for the evaluation of binding parameters. In Equation 2, n is the number of binding sites for RBP per molecule of TTR, C_{RBP} is the concentration of free RBP, nC_{TTR} and $C_{RBP\cdot TTR}$ are concentrations of binding sites on TTR, respectively, free and complexed with RBP.

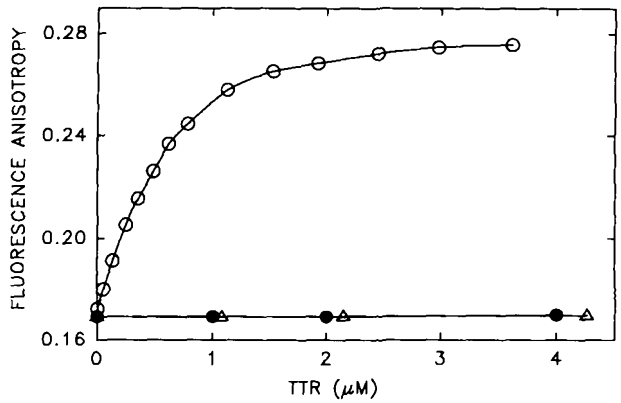

FIG. 1. **Titrations of holoRBP with recombinant normal and variant TTRs.** Fluorescence anisotropy of human holoRBP is plotted as a function of the concentrations of recombinant human normal TTR (○), of recombinant Ser-84 TTR (●), and of recombinant Ser-84 TTR preincubated with a 2-fold molar excess of L-thyroxine (△). Conditions consisted of 1 μM holoRBP in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl, at 20 ± 0.5 °C. Retinol fluorescence was monitored at 460 nm with excitation at 330 nm. The cuvette path length was 10 mm.

Equation 2 can be written in the form shown in Equation 3, where α is the fraction of free RBP, C_{oRBP} and C_{oTTR} are the total RBP and TTR concentrations, respectively.

$$C_{oRBP}\alpha = [C_{oTTR}\, n\alpha/(1 - \alpha)] - K_d \qquad \text{(Eq. 3)}$$

If the binding sites for RBP on TTR are equivalent and independent, a plot of $C_{oRBP}\,\alpha$ *versus* $C_{oTTR}\,\alpha/(1-\alpha)$ will give a straight line with slope and intercept on the ordinate axis corresponding to n and K_d, respectively. The value of α was calculated for every point of the titration curve using the relation shown in Equation 4, where A_{max} and A_o are the two limiting fluorescence anisotropy values for RBP-bound retinol, *i.e.* in the presence of an excess saturating TTR and in the absence of TTR, respectively; A represents the fluorescence anisotropy value for a certain total concentration of TTR.

$$\alpha = (A_{max} - A)/(A_{max} - A_o) \qquad \text{(Eq. 4)}$$

An assumption of the above method is that the fluorescence anisotropies of RBP·TTR complexes with stoichiometries of binding higher than one have the same anisotropy values as the 1:1 complex. This assumption appears to be valid, as the Stokes molecular radii of RBP·TTR complexes exhibiting 1:1 and 2:1 stoichiometries are identical (18). The method of Kopelman *et al.* (17) is particularly useful because it does not require, for the evaluation of binding parameters, the concentration of free ligand, *i.e.* the concentration of free binding sites on the tetrameric TTR molecule, but instead, the total molar concentration of TTR present in the system.

RESULTS AND DISCUSSION

Interaction of Recombinant Normal TTR with RBP—The addition of TTR to holoRBP causes a substantial increase in the fluorescence anisotropy of RBP-bound retinol. Such increase may be attributed to the modification of the rotational diffusion behavior of holoRBP upon formation of the RBP·TTR complex, which has a remarkably higher molecular mass as compared with uncomplexed holoRBP. A typical corrected curve for the titration of human RBP with recombinant human normal TTR is shown in Fig. 1. A satisfactorily good fitting of the data to a straight line was obtained by plotting them according to the method of Kopelman *et al.* (17) (Fig. 2). From the slope and intercept on the ordinate axis, 1.9 RBP binding sites/molecule of TTR and a dissociation constant of 0.45 μM were determined. The fitting of the data to a straight line and the estimated stoichiometry of approximately two sites for RBP per TTR molecule indicate that RBP binds simultaneously to two equivalent and independent sites on TTR. A stoichiometry of 1.5 binding sites for RBP on TTR and

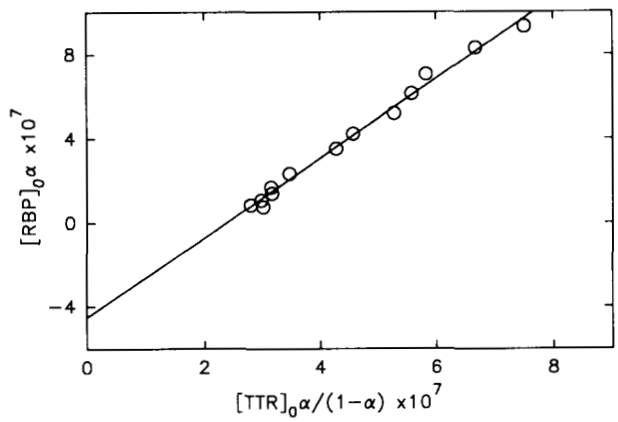

FIG. 2. **Analysis of binding data.** Linear least squares plot of $[RBP]_0\alpha$ *versus* $[TTR]_0\alpha/(1-\alpha)$, for the titration of human holoRBP with recombinant human normal TTR shown in Fig. 1. $[RBP]_0$ and $[TTR]_0$ are the total molar concentrations of RBP and TTR in the system; α is the fraction of free RBP.

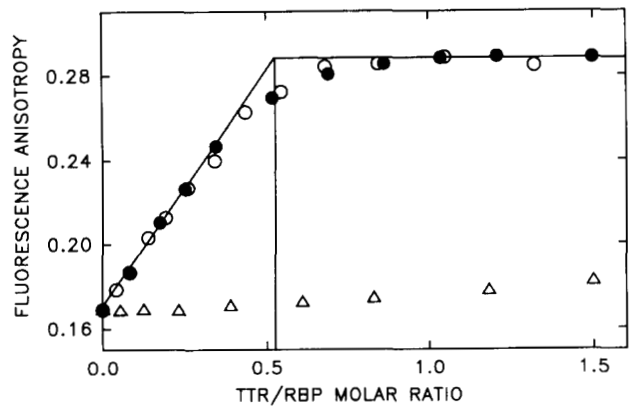

FIG. 3. **Stoichiometric binding of RBP to TTR.** Fluorescence anisotropy of human holoRBP is plotted as a function of the TTR/RBP molar ratio. ●, TTR purified from human plasma; ○, recombinant human normal TTR; △, recombinant Ser-84 TTR. Conditions are the same as for Fig. 1, except for RBP concentration (20 μM). The cuvette path length was 3 mm.

a rather low dissociation constant (0.07 μM) were found in a previous study employing a chemically modified form of bovine TTR (14). Instead, the results reported here compare well enough with those obtained in a comparative study of the binding properties of human and bovine RBPs and TTRs, where the same experimental conditions and the same method of analysis of binding data as in the present investigation were adopted (19). The only difference between these two sets of data concerns the value of the dissociation constant, which for recombinant human TTR is 1.5–2-fold higher than that for TTR purified from human plasma.

Since conflicting data regarding the binding stoichiometry of RBP to TTR have been reported previously, it appeared to be of interest to further investigate this issue for both plasma and recombinant TTRs. The most direct method of evaluating the number of RBP binding sites per molecule of TTR is that of performing titrations using concentrations of interacting proteins substantially higher than the dissociation constant, thus promoting stoichiometric binding. When such an approach was used and TTR was added to holoRBP at a concentration of 20 μM, *i.e.* approximately 50-fold higher than the above reported dissociation constant, a virtually linear increase in fluorescence anisotropy was found (Fig. 3). The number of RBP binding sites per TTR molecule can be estimated from the intersection point of the linear rise of fluorescence anisotropy and its plateau value at saturating concentrations of TTR. Since the intersection point corresponds to a TTR/RBP molar ratio of about 0.5, this direct binding assay clearly indicates the presence of two sites for RBP on both plasma and recombinant TTRs. These data may thus be regarded as a proof of the validity of the estimates of binding parameters we have obtained using the method of Kopelman *et al.* (17).

Lack of Interaction of the Ser-84 Variant of TTR with RBP—In the Ser-84 variant of human TTR the Ile residue at position 84 of normal TTR is replaced by Ser. When Ser-84 TTR was added to human holoRBP, no significant increase in fluorescence anisotropy of the RBP-bound retinol was revealed under the conditions adopted to study the interaction with RBP of recombinant normal TTR (Fig. 1). We have also addressed the possibility that structural alterations in the TTR molecule induced by the Ile-84 → Ser amino acid substitution and possibly affecting the interaction with RBP could be reverted by the binding of L-thyroxine. To this end, a titration was carried out in which Ser-84 TTR was incubated with a 2-fold molar excess of thyroxine prior to the addition to holoRBP. It should be noted that the above thyroxine concentrations are practically saturating on the basis of the reported dissociation constant for the Ser-84 TTR (28). Indeed, no significant influence of the preincubation with thyroxine on the interaction of the variant TTR with RBP could be revealed (Fig. 1). To investigate a wider range of interacting protein concentrations, the titration experiment shown in Fig. 3, which used concentrations of RBP and TTR substantially higher compared with the titrations presented in Fig. 1, was also carried out with Ser-84 TTR. Only a very limited increase in fluorescence anisotropy of retinol-RBP could be detected in the presence of 20 μM RBP and of Ser-84 TTR up to 30 μM (Fig. 3). Therefore, Ser-84 TTR appears to display a quite low affinity, if any, for RBP. One might argue that a nearly negligible binding of Ser-84 TTR to RBP may be the result of profound alterations of the TTR structure caused by the Ile-84 → Ser amino acid substitution. However, Ser-84 TTR displays an affinity for thyroxine only 2–3-fold lower than that of normal TTR (28, 29). Therefore, the binding sites for thyroxine in the TTR central channel are likely to be preserved. Furthermore, the crystal form of the Ser-84 variant of TTR is isomorphous to that of normal TTR, indicating that no gross alterations of the TTR three-dimensional structure are induced by the Ile-84 → Ser substitution (33). Position 84 is located on the outer surface of the TTR molecule, in the turn connecting the short α-helix with the N terminus of β-strand F (5); therefore, the area comprising Ile-84 is a candidate for the interaction with RBP. Interestingly, a significant conformational change in the above area on the protein surface has been revealed in the crystal structure of Ser-84 TTR as compared with normal TTR.[2] The drastically reduced affinity of Ser-84 TTR for RBP we have observed might be explained on a structural basis. In fact, the above local conformational change in Ser-84 TTR is large enough to justify the interference with protein-protein recognition and, therefore, probably involves the RBP binding site of TTR. In conclusion, the results of this study coupled with other lines of evidence strongly suggest that a region on the outer surface of the TTR molecule comprising Ile-84 participates in the interaction with RBP. It is worth noting that a recently presented hypothetical molecular model for the RBP·TTR complex, based on a computer graphics docking procedure, is consistent with the involvement of a TTR region comprising Ile-84 in the binding of RBP and with the presence of two binding sites for RBP on TTR (34). This model also predicts the participation of some of the β-barrel entrance loops in RBP in the interaction with TTR. However, the model

[2] J. A. Hamilton, personal communication.

does not fully account for the results of a structural study with a retinoid-RBP complex lacking affinity for TTR, which suggest a direct participation of the opening of the β-barrel cavity in the RBP-TTR recognition (16).

It has been established previously that plasma RBP concentrations are unusually low (≈30% of the normal values) in carriers of the amyloidogenic Ile-84 → Ser mutation (25, 26). To explain this finding, it was proposed that the Ser-84 TTR may display an altered affinity for RBP, thus interfering with the normal transport of the vitamin A plasma carrier. Our data provide a direct experimental support for the above suggestion and further stress the importance of the RBP·TTR complex in RBP metabolism. Experimental evidence has already been presented to indicate the relevant physiological role of the RBP·TTR complex. In particular, the lack of the RBP·TTR complex formation in TTR-deficient mice is responsible for a reduction in plasma RBP concentration to about 3% of the normal value (35). An approximately 30-fold reduction in plasma RBP for TTR-deficient mice is not surprising when compared with the 3-fold reduction found in carriers of the amyloidogenic Ile-84 → Ser mutation. In fact, it should be noted that the individuals carrying the above mutation are essentially heterozygous (36) and their "heterozygous" TTR, which consists of 35–40% of Ser-84 TTR and of 60–65% of normal TTR, is expected to possess to some extent the ability to form complexes with RBP. Therefore, a lower degree reduction in plasma RBP is also expected for such individuals as compared with TTR-deficient mice, in which the RBP·TTR complex is completely lacking. Moreover, high doses of retinoids exhibiting *in vitro* affinity for RBP and whose binding to the plasma retinol carrier interferes with the formation of the RBP·TTR complex also cause a substantial early reduction in plasma RBP (37). The reduced or abolished formation of the RBP·TTR complex might lead to the lowering of plasma RBP by interfering with the secretion of RBP from the liver, as suggested previously (37), and/or by determining an accelerated filtration of uncomplexed RBP through kidney glomeruli (3).

Finally, we have also considered a possible correlation between the amyloidogenicity of point mutations in TTR and the interference with RBP-TTR recognition. In preliminary studies, the Ile-84 → Ser substitution in TTR was the only one, among several amyloidogenic amino acid substitutions, capable of drastically affecting the interaction with RBP.[3] Therefore, a drastically altered affinity of TTR for RBP appears to be an independent and distinct property of the Ser-84 variant of TTR rather than a general feature associated with familial amyloidotic polyneuropathy.

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[3] G. Malpeli, C. Folli, and R. Berni, unpublished observations.

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