ARTICLE

ACS chemical biology

Retinol and Retinol-Binding Protein Stabilize Transthyretin *via* Formation of Retinol Transport Complex

Suk-Joon Hyung[†,‡,*], Stéphanie Deroo[†], and Carol V. Robinson[†]

[†]Department of Chemistry, Oxford University, South Parks Road, Oxford, OX1 3QY, United Kingdom and [‡]University of Michigan, Department of Chemistry, 930 North University, Ann Arbor, Michigan 48109, United States

ABSTRACT Transthyretin (TTR) is a plasma hormone carrier protein associated with hereditary and senile forms of systemic amyloid disease, wherein slow tetramer disassembly is thought to be an obligatory step. Plasma transport of retinol is carried out exclusively by the retinol-binding protein (RBP), through complexation with transthyretin. Using mass spectrometry to examine the subunit exchange dynamics, we find that retinol stabilizes the quaternary structure of transthyretin, through its interactions with RBP, reducing the rate of transthyretin disassembly ~17-fold compared to apoTTR. In the absence of retinol but in the presence of RBP, transthyretin is only marginally stabilized with the rate of disassembly reduced ~two-fold with respect to apoTTR. Surprisingly, we found two retinoids that stabilize transthyretin directly, in the absence of RBP, whereas retinol itself requires RBP in order to stabilize transthyretin. Our results demonstrate new roles for RBP and retinoids as stabilizers of transthyretin.

Retinoids (vitamin A and its analogues) are capable of a number of biological functions, including roles in embryonic development and homeostasis, through regulation of their target genes *via* activation of the nuclear retinoid receptor (*1*). Transport of all-trans retinol (vitamin A) from the liver to the peripheral tissues takes place in the plasma with the ligand bound to a specific carrier protein, retinol-binding protein (RBP) (*2*). It is established that RBP confers stability to retinol as well as serves as a vehicle for solubilizing hydrophobic retinol (*2, 3*). Retinol regulates the secretion of the RBP into plasma, since in its absence RBP accumulates in the endoplasmic reticulum of hepatocytes and is only released upon the restoration of cellular levels of retinol (*4*). A number of retinoids in addition to retinol are present in plasma at nanomolar concentrations (5–10 nM) including all-trans retinoic and 9-cis retinoic acids (*5, 6*). These retinoids are thought to be transported to target cells using the common lipoproteins in plasma (*7, 8*). These retinoids are biologically active forms of retinol in the target cells, with no known functional roles in plasma.

The majority of RBP (~95%) circulates in plasma in complex with another plasma protein transthyretin (TTR), which also functions as an exclusive carrier of the thyroxine hormone in cerebrospinal fluid (*9*). Retinol binding induces subtle conformational changes in RBP which leads the RBP–all-trans retinol complex (holoRBP, 21 kDa) to bind to TTR with higher affinity than apoRBP, thereby forming the retinol transport complex (*10*). The normal RBP concentration in plasma is about 2 μM (*11*), and that of TTR is about 3.6–4.5 μM (*12*), suggesting that up to 50% of the TTR in plasma may be associated with holoRBP, which binds to TTR

*Corresponding author, carol.robinson@chem.ox.ac.uk.

Received for review May 22, 2010 and accepted September 16, 2010.

Published online September 16, 2010

10.1021/cb100144v

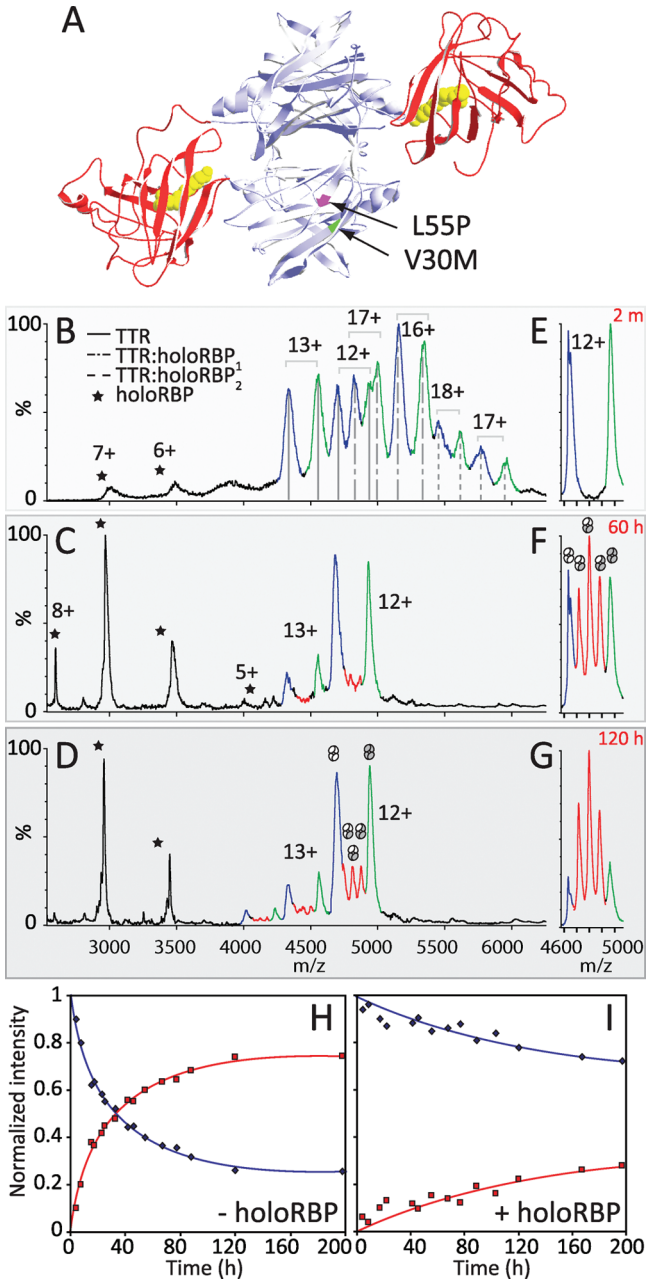

Figure 1. HoloRBP moderates the dynamics of the TTR tetramer. A) The X-ray structure of human TTR:holoRBP$_2$ complex. TTR is shown in blue, RBP is in red, and retinol is in yellow. The position of L55P and V30 M point mutations are indicated in purple and green, respectively. The structure was produced from the PDB code 1QAB using Swiss-PDB viewer. Mass spectra of the subunit exchange reaction of [^{12}C–^{14}N]TTR and [^{13}C–^{15}N]TTR: B–D) with holoRBP and E–G) without RBP is shown after: B and E) 2 min, C and F) 60 h, and D and G) 120 h. Peaks assigned to the TTR tetramer containing only [^{12}C–^{14}N] subunits are shown in blue, [^{13}C–^{15}N] subunits are shown in green, and hybrid TTR tetramers containing 1–3 [^{12}C–^{14}N] and 1–3 [^{13}C–^{15}N] subunits are shown in red. H and I) Relative intensity of the homotetramer (blue and green) against the heterotetramers (red) is plotted against time. Data are compared with the intensities predicted from the model (solid lines).

with high affinity (1.5×10^{-7} M) (*13*). TTR presents four symmetry-related RBP-binding sites; however a maximum of two RBP bind simultaneously *in vitro* due to the proximity of the binding sites (Figure 1, panel A).

In its native state, TTR is stable as a 56 kDa homotetramer organized as a dimer of dimers (*14*). Unusually for a multiprotein complex, which under physiological conditions is normally folded with a well-defined globular structure, TTR tetramer can disassemble into monomers that can partially unfold and misassemble into amyloid fibrils (*15–17*). Subsequent deposition of insoluble and thermodynamically stable fibrils can manifest pathologically as senile systemic amyloidosis (SSA) or familial amyloidotic polyneuropathy (FAP), the latter of which is a hereditary form of TTR amyloidosis. Over 80 different point mutations that result in the pathology of FAP are known, including V30 M and L55P, the most common and most aggressive point mutations of TTR, respectively (*18*).

The subunit dynamics of the TTR tetramer under nondenaturing solution conditions can be elucidated by monitoring the course of the subunit exchange process, which consists of slow disassembly of TTR tetramer to monomer, prior to rapid reassembly (*19*). The subunit exchange of V30 M and L55P variant TTR tetramers proceeds over a wide range of time scales, with L55P exchanging more rapidly than wild-type (WT) or V30 M (*19, 20*). This indicates a correlation between the amyloidogenicity of TTR and the rate-limiting disassembly of the TTR tetramer required for TTR amyloid fibril formation. Previously, acid-induced formation of TTR amyloid fibrils was shown to diminish upon establishing the retinol transport complex (*21*). The protein–protein contacts between holoRBP and three subunits of TTR, across both dimer interfaces, were proposed as the key interactions that stabilize the TTR tetramer (*22*). Mass spectrometry (MS) experiments of the intact retinol transport complex were also able to demonstrate that holoRBP stabilizes TTR (*13*). To date, all studies of the dynamics of TTR have been conducted in the absence of RBP or retinol. Thus the dynamics within the physiological lifetime (~24–48 h), during which time TTR is reallocated into a higher-order retinol transport complex, are not understood in molecular detail.

We previously elucidated the subunit exchange dynamic of TTR by means of a MS-based approach that is capable of identifying the composition of intact protein complexes within heterogeneous systems (*20*). Specifi-

cally, TTR is uniformly labeled with stable isotopes (^{13}C and ^{15}N) to provide sufficient mass differences to resolve heterocomplexes that contain both unlabeled and labeled subunits by MS. This labeling strategy does not perturb interactions with RBP, rendering the method suitable for assessing the influence of RBP on the dynamics of TTR. The capacity of MS to identify protein complexes under equilibrium conditions, where multiple-binding states coexist, allows quantitative determination of intact protein complexes with different stoichiometries, in real-time (*23–25*). Here we measure the course of subunit exchange of TTR tetramers within the retinol transport complex and show that the disassembly of TTR tetramers is reduced upon formation of this complex in a concentration-dependent manner. We found that the dynamics of TTR were affected significantly by addition of retinol in concert with RBP. This was evidenced by a remarkable range of stabilities of WT TTR and two FAP-associated variants (V30 M and L55P). Further, the discovery of several retinoids that are able to stabilize the TTR tetramer even in the absence of RBP highlights the role of these retinoids in inhibiting the disassembly of TTR tetramers thus possibly ameliorating the early steps of TTR amyloid fibril formation.

RESULTS AND DISCUSSION

Subunit Exchange Dynamics of TTR with holoRBP. To investigate the potential of RBP to influence the disassembly of TTR, the dynamics of TTR quaternary structure and the retinol transport complex were studied under physiological pH and temperature conditions (37 °C; pH 7.0). Using both unlabeled and labeled TTR, incubated with stoichiometric concentrations of holoRBP, we recorded mass spectra as a function of time (Figure 1, panels B–G). The mass spectrum of an equimolar solution of unlabeled and labeled TTR shortly after addition of holoRBP shows a charge-state series that corresponds to two different RBP-bound and -unbound states of TTR, both unlabeled and labeled (Figure 1, panel B). Individual species containing tetrameric TTR were identified by their unique masses (Supplementary Table S1). The intensity of the retinol transport complex containing unlabeled TTR is very similar to that containing labeled TTR, demonstrating that isotopic labeling of TTR has no impact on the binding of holoRBP to TTR. The broad peak width is consistent with the "soft" ionization conditions used here to maintain protein–protein interactions between TTR and holoRBP (*26*) and is considered to reflect residual water and buffer ions. In order to reduce the spectral complexity that arises from heterogeneous nature of the system, the retinol transport complex ions were activated to effect dissociation of holoRBP as well as the water and buffer ions. After 60 h, the mass spectrum of the solution, containing unlabeled and labeled TTR with holoRBP, was acquired under conditions designed to dissociate the retinol transport complex into tetrameric transthyretin in the gas phase. The intensity of peaks corresponding to RBP (+6 to +8) is increased significantly compared to that assigned to free TTR, and the peaks corresponding to TTR in complex with holoRBP are not observed. This indicates that TTR is primarily in its unbound form in the gas phase under these activation conditions (Figure 1, panel C). This approach permits sufficient resolution to identify fine detail within the MS peaks, as demonstrated in the m/z region between 4600 and 5000 Da (Figure 1, panel C, inset) wherein new peaks are assigned to heterotetramers containing both unlabeled and labeled TTR subunits (Supplementary Table S2). Changes in the spectra over time reveal the progress of TTR subunit exchange, with the peaks corresponding to the heterotetramers increasing in intensity relative to homotetrameric TTR (Figure 1, panels C and D). After 120 h, a significant quantity of homotetrameric TTR remains, with only small peaks corresponding to heterotetramers. This contrasts remarkably with the control reaction, without holoRBP, where heterotetramers are dominant after 120 h at which time the reaction is effectively complete (Figure 1, panels E–G).

The normalized intensity of peaks corresponding to TTR homotetramers and heterotetramers showed a marked difference in the progression of subunit exchange between the control experiment and that with holoRBP (Figure 1, panels H and I, respectively). This demonstrates that the dynamics of the TTR quaternary structure can be retarded significantly by the binding of holoRBP. We assessed the dynamics of TTR quaternary structure quantitatively, by simulating the loss of homotetramers using a mathematical model that involves both the disassembly of TTR tetramers into monomeric or dimeric TTR and their reassembly into TTR tetramers (*20*); the latter was assumed to be much faster than the disassembly of the tetramer since the disassembly of TTR tetramer is the time-limiting step (*19*) (Supplementary Figure S1). The simulated subunit exchange profiles

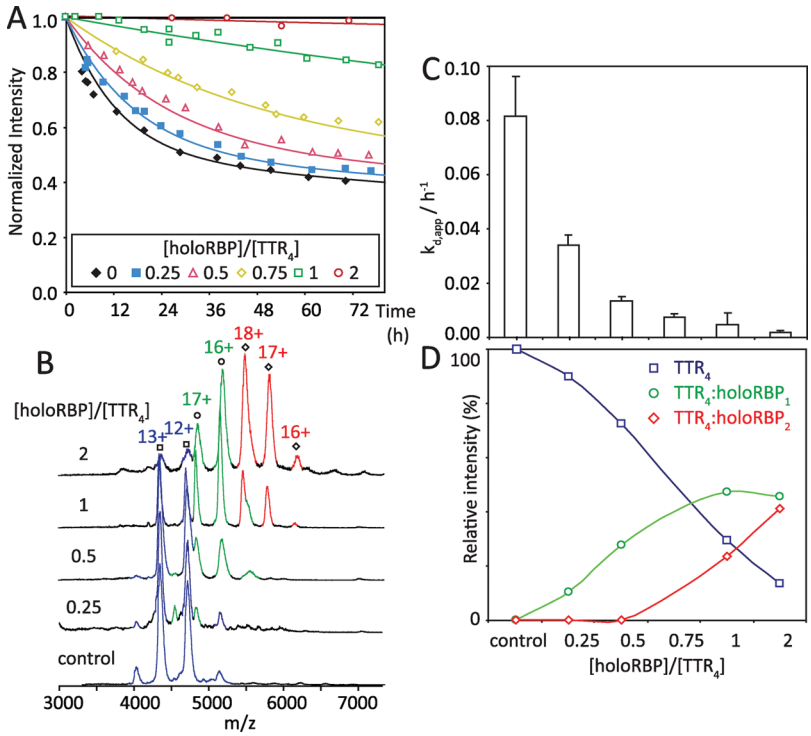

Figure 2. Formation of the retinol transport complex reduces the dynamics of TTR. A) Decay of the TTR homotetramer at increasing concentrations from 0 (black filled diamond) to 2 mol equiv of holoRBP to TTR (red open circle). B) Mass spectrum of TTR incubated with increasing concentrations of holoRBP for 2 min. Peaks corresponding to TTR (blue), TTR:holoRBP$_1$ (green), and TTR:holoRBP$_2$ (red) are annotated. C) Plot showing the rate of TTR disassembly ($k_{d,app}$) with the error bars indicating the standard deviation of three triplicate samples. D) Equilibrium concentrations of free TTR tetramer (blue square), TTR:holoRBP$_1$ (green circle), and TTR:holoRBP$_2$ (red diamond) at holoRBP concentrations ranging from 0 to 2 equiv of TTR.

of the decay of homotetrameric TTR and the production of the heterotetramer are most consistent with the experimental data when the rate of disassembly ($k_{d,app}$) of TTR with holoRBP reduces substantially to 0.005 h^{-1}, compared to the control experiment with the corresponding rate of 0.082 h^{-1}. This demonstrates the accuracy of the model in recapitulating the subunit exchange process (Figure 1, panels H–I, red and blue lines). Importantly the results also show that binding of holoRBP stabilizes the quaternary structure of TTR.

Formation of the Retinol Transport Complex Stabilizes the TTR Tetramer. To determine the concentration dependence of the reduced dynamics of the TTR quaternary structure in the retinol transport complex, the course of subunit exchange of TTR was monitored under increasing concentrations of holoRBP. As the concentration of holoRBP was raised from 0 to 2 mol equiv of TTR, homotetramers were found to decay more slowly (Figure 2, panel A). This clearly demonstrates a concentration-dependent inhibition of subunit exchange by holoRBP. It is also possible to calculate the relative abundance of individual RBP-bound states of TTR from mass spectra of TTR at varying concentrations of holoRBP. This indicates that the equilibrium between free TTR and the retinol transport complex shifts toward the latter with increasing concentration of holoRBP (Figure 2, panel B). Notably, the rate of TTR disassembly demonstrated a direct correlation with the relative abundance of free TTR (Figure 2, panels C and D). The abundance of the retinol transport complex with one or two copies of holoRBP showed a correlation with the rate of TTR disassembly, indicating that the formation of the retinol transport complex stabilizes the TTR tetramer. Importantly, the rate of TTR disassembly is substantially reduced by stoichiometric concentrations of RBP. For example, $k_{d,app}$ is reduced >17-fold with a stoichiometric quantity of RBP, when TTR exists mainly as a retinol transport complex carrying only one copy of holoRBP (Figure 2, panel C, Supplementary Table S3). This suggests that a single holoRBP molecule is sufficient to stabilize the tetrameric assembly and retard subunit exchange significantly. This is similar to the observation made for small molecule binding to TTR, in that occupation of a single thyroxine-binding site by a ligand is sufficient to stabilize the TTR tetramer (*27*). These data provide direct evidence, therefore, that WT TTR is stabilized under conditions that allow oc-

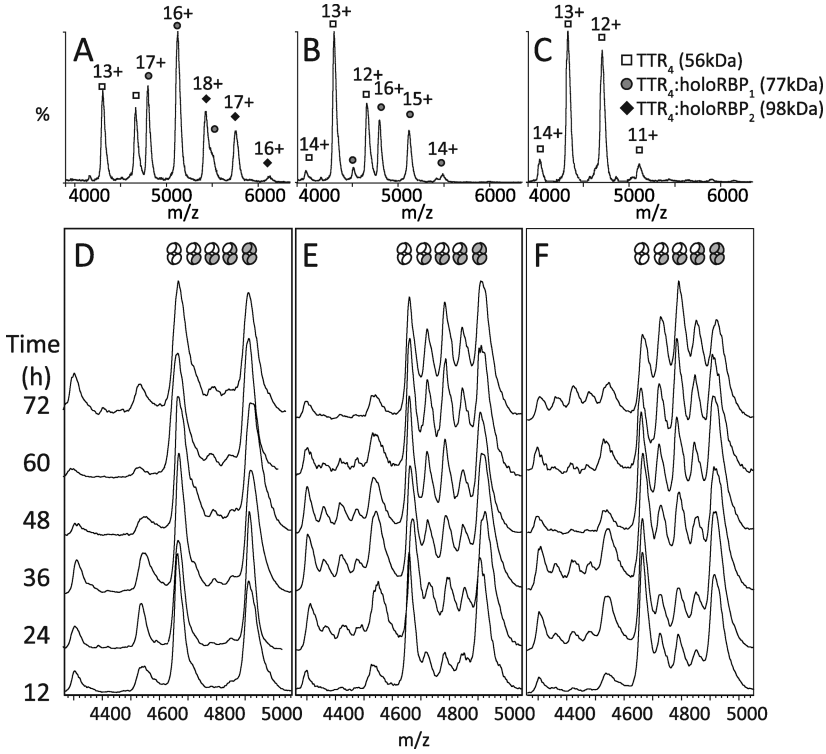

Figure 3. The effect of retinol on the dynamics of TTR in the retinol transport complex. Mass spectrum of TTR with an equimolar concentration of: A) holoRBP, B) apoRBP and C) TTR alone. Subunit exchange data of WT [^{12}C–^{14}N]TTR and WT [^{13}C–^{15}N]TTR with D) holoRBP, E) apoRBP, and F) without RBP. Mass spectra acquired under dissociation MS conditions at a fixed time interval showing the decay of homotetrameric TTR and the evolution of heterotetramers over time.

cupancy of one or both RBP-binding sites to form the stable retinol transport complex.

Retinol Binding Modulates Dynamics of TTR and Its Variants. The identification of retinol transport complexes that stabilize TTR, along with the observation that these complexes are populated at equilibrium, provides an opportunity to determine the potential of retinol to mediate binding of RBP to TTR and consequently to affect the stability of the TTR tetramer. We studied TTR incubated either with a stoichiometric concentration of holoRBP or with RBP only (*i.e.*, apoRBP) under non-denaturing conditions at neutral pH. In the presence of holoRBP three well resolved charge-state series are observed between 4000 and 6500 *m*/*z*, corresponding to intact TTR, TTR:holoRBP$_1$, and TTR:holoRBP$_2$ with TTR:holoRBP$_1$ as the dominant species (Figure 3, panel A). By contrast, in the presence of apoRBP binding of two apoRBP per TTR is not observed, the major species observed in the spectrum being free TTR (Figure 3, panel B, compare with 3C). This confirms the weaker binding affinity of apoRBP to TTR compared to holoRBP. In the light of our observation that the abundance of free tetramer is associated with the dynamics of the TTR quaternary structure (Figure 2, panel C), we anticipated that TTR with apoRBP would undergo subunit exchange more rapidly than TTR:holoRBP.

To test this hypothesis we studied subunit exchange between unlabeled and labeled TTR homotetramers with either holoRBP or apoRBP. In the presence of apoRBP a substantially higher abundance of heterotetramers was formed over the course of 72 h compared to that formed in the presence of holoRBP. Heterotetramers formed from TTR alone were however at a higher abundance than those formed in the presence of apoRBP, leading to a disassembly rate of TTR in the order of TTR + holoRBP < TTR + apoRBP < TTR (Figure 3, panels D–F, respectively). We also found that the rate of decay of homotetramers is reduced only marginally by apoRBP compared to that of the control experiment (Figure 4, panel A). Addition of retinol to TTR and apoRBP however decreases the concentration of free TTR, which is competent to exchange subunits (Figure 3, panel A). Consequently the rate of subunit exchange is further reduced relative to that of TTR alone. Overall the results show that the WT protein holoRBP is more effective in inhibiting subunit exchange than apoRBP.

Having established these differences with WT TTR we now turn our attention to two TTR variants: V30 M and L55P. These proteins were found to establish reti-

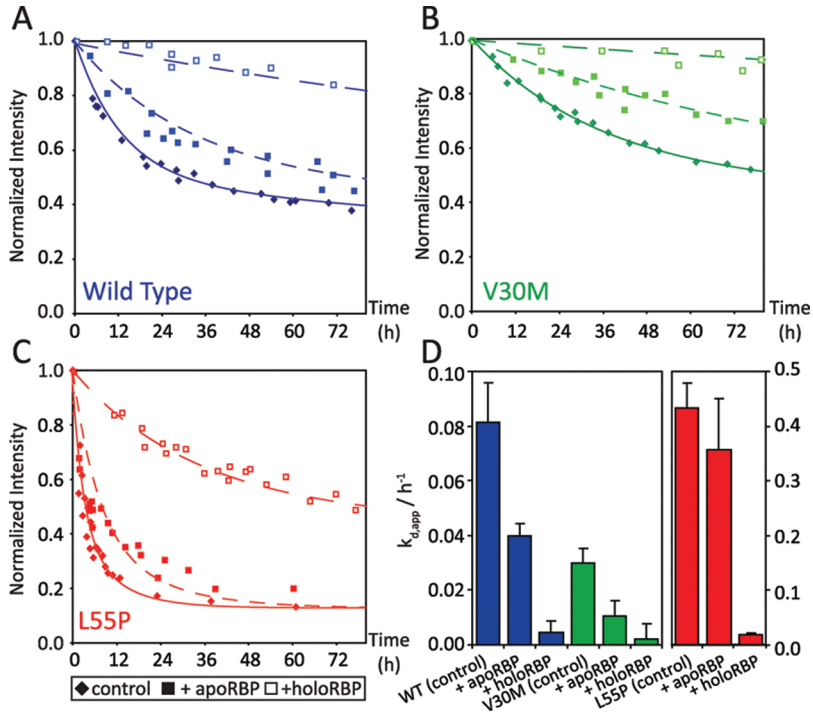

Figure 4. The influence of point mutations on the dynamics of transthyretin in the apo- and holo-retinol transport complex. The decay of homotetramer against time determined from subunit exchange reactions with: A) WT, B) V30 M, and C) L55P TTR is shown for subunit exchange with holoRBP (blank square), apoRBP (solid square), and TTR alone (filled diamond). D) Rate of disassembly of TTR tetramer in each system is shown with the error bars indicating the standard deviation of three triplicate samples.

nol transport complexes with similar affinity compared to WT protein (Supplementary Figure S2). This suggests that these disease-associated point mutations of TTR do not compromise the binding of RBP (*13*). For the V30 M variant, a similar cumulative slowing of subunit exchange was observed from the binding of apoRBP and holoRBP to TTR (Figure 4, panel B, Supplementary Figure S3). For the L55P variant, the TTR tetramer itself is significantly more dynamic, and subunit exchange proceeds more rapidly, and the effects of binding holoRBP are more marked compared with WT or V30 M TTR (Figure 4, panel C, Supplementary Figure S3). The rate of tetramer disassembly in each system is compared and highlights a remarkably wide range in $k_{d,app}$ (Figure 4, panel D). L55P TTR undergoes subunit exchange most readily (0.448 h^{-1}), followed by WT TTR and V30 M TTR (0.082 and 0.030 h^{-1}, respectively), in agreement with the order determined previously (*19, 20*). Interestingly, the dynamics of L55P variant TTR tetramer are only marginally retarded by apoRBP binding compared to WT TTR, and $k_{d,app}$ is reduced to ~85 and ~50% of the control respectively (Figure 4, panel D). Moreover, the stability of L55P TTR increased substantially with subsequent addition of retinol compared to WT and V30 M TTR ($k_{d,app}$ decreasing by ~18-, ~8-, and ~5-fold for L55P, WT, and V30 M TTR, respectively). These data demonstrate, therefore, that differential stability can be afforded to variants of TTR by apoRBP and retinol.

Retinoids Modulate the Dynamics of TTR. Having established that retinol in concert with RBP can stabilize TTR, we explored the capacity of a range of retinoids to stabilize TTR by themselves or through binding to RBP, including the active metabolites (retinol, all-trans retinoic acid, 9-cis retinoic acid, and retinal) as well as a natural ester of retinol (retinyl acetate) (Figure 5, panel A). The ability of equimolar concentrations of different retinoids to stabilize tetrameric TTR (4.5 μM retinoids and WT TTR in the absence of RBP) was evaluated by monitoring the dynamics of TTR quaternary structure. Retinol, retinal, and retinyl acetate did not affect significantly the disassembly of TTR at the concentration of retinoids tested (Figure 5, panel B). By contrast, in the presence of retinoic and 9-cis retinoic acids, TTR exhibited significantly reduced quaternary dynamics indicating that these retinoids are able to retard subunit exchange in TTR tetramers, surprisingly even in the absence of RBP. To establish the mode of tetramer stabilization by retinoic acid, we analyzed TTR incubated with both thyroxine and retinoic acid to determine whether or not both molecules could bind simultaneously. TTR was found to be competent to bind retinoic acid and thyroxine together at 1:1

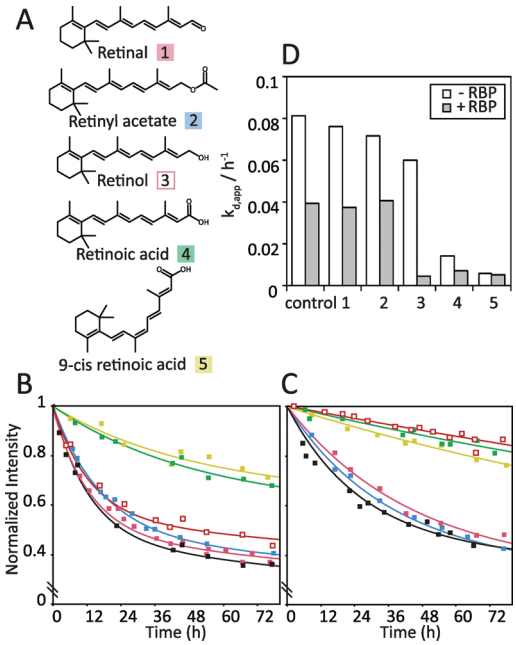

Figure 5. TTR stabilization in the presence of retinoids. A) Structures of retinal (1, pink), retinyl acetate (2, blue), retinol (3, red), retinoic acid (4, green), and 9-cis retinoic acid (5, yellow). Comparison of the ability of each retinoid to retard disassembly of WT TTR with: B) retinoids only and C) with addition of retinoid and apoRBP, shown in both cases with control experiments in the absence of retinoids (black). D) The rate of disassembly is shown for each experiment.

stoichiometry (Supplementary Figure S4), showing clearly that retinoic acid binds directly to TTR. Interestingly, retinoic acid was not found to bind to TTR when two thyroxine ligands were bound, suggesting a competition between retinol and thyroxine for binding sites (Supplementary Figure S4). However binding of the retinoic acid:RBP complex to TTR was found to be independent of thyroxine binding (data not shown). These results imply that in the absence of RBP, retinoic acid binds in the thyroxine-binding sites of TTR to stabilize the tetramer from disassembly.

Addition of equimolar concentrations of apoRBP to the TTR:retinoid complexes was found to slow subunit exchange in all cases (Figure 5, panel C). Plots of the progress of the reaction show that both retinal and retinyl ester mirror the subunit exchange profile of the control experiment with apoRBP only. By contrast, the rate of TTR disassembly is significantly reduced upon addition of RBP to the TTR and retinol. This demonstrates that retinol renders RBP competent to stabilize TTR substantially. Both retinoic and 9-cis retinoic acids, with or without RBP, effectively stabilize TTR leading to a rate of disassembly that is comparable to that of holoRBP (retinol–RBP).

Discussion. TTR adopts a dynamic tetrameric structure whose subunits disassemble–reassemble to produce a pool of subunits/subcomplexes that serve as a putative starting point for amyloid fibril formation (*15, 16, 28*). The slow disassembly of the TTR tetramer thus represents a rate-limiting step for TTR amyloidosis within the physiological half-life of TTR (24–48 h) (*19, 29*). Despite speculation that the course of TTR amyloidosis may be regulated by RBP and retinol (*21, 30*), clear evidence for their role in the dynamics of the TTR was not evident. Here, the use of electrospray ionization mass spectrometry (ESI-MS) has allowed us to assess the formation of the intact retinol transport complex as well as to monitor the subunit exchange of TTR with RBP under nondenaturing conditions and to assess the subunit exchange dynamics of TTR. Our results show that TTR incubated with holoRBP undergoes disassembly at a reduced rate, in a concentration-dependent manner through the formation of a stable retinol transport complex. Consequently this may have an implication for the dynamics of the TTR *in vivo* wherein up to 50% of TTR complexes in plasma are associated with RBP. In the presence of physiological concentrations of holoRBP, TTR disassembles at a rate which is approximately sixfold slower than that of TTR alone. Therefore we can speculate that the normal abundance of holoRBP can retard significantly the disassembly of TTR. We have also demonstrated that apoRBP can bind to TTR, albeit at lower affinity compared to holoRBP, affording some level of stabilization to TTR through formation of the TTR:apoRBP complex analogous to the retinol transport complex. The existence of this complex at physiological concentrations in the plasma has not been established (*31*). The assembly of such a complex however would provide a mechanism to retain RBP in plasma by increasing the molecular weight and preventing molecular filtration in the kidney, even when retinol levels are depleted.

The role of retinol in modulating the stability of TTR is further highlighted by the ability of retinol to stabilize TTR, in concert with RBP, more significantly than all other retinoids examined here. This indicates that TTR elicits significantly higher binding affinity for RBP bound to reti-

nol compared to other retinoids. The result is consistent with the function of RBP as an exclusive carrier of retinol in plasma. Surprisingly, we found that two retinol metabolites detected in plasma *in vivo* (retinoic and 9-cis retinoic acids) stabilize TTR directly, without RBP. By contrast, those that are primarily localized in the eye (retinal) or that are readily modified before absorption into enterocytes upon intake (retinyl acetate) were not able to stabilize TTR. It is interesting to note that these active retinoids possess carboxyl groups that could enable the necessary electrostatic interactions with the outer binding site of the thyroxine-binding channel to stabilize the tetrameric state. Since small inhibitor molecules that stabilize tetrameric TTR are known to be effective at inhibiting the formation of amyloid fibrils, these active retinoids are likely to exhibit a similar inhibitory effect. The low plasma concentration of these active retinoids with respect to the concentration of TTR however would mean that stabilization of TTR could only be elicited in response to high plasma levels of these metabolites, upon large intake of vitamin A (*6*).

Therefore, the present study brings into focus the importance of understanding the TTR disassembly in the context of the retinol transporter. The disassembly incompetent and the native forms of TTR can be partitioned and regulated by RBP or retinol. As a consequence of the complicated interplay between retinol and RBP, an extremely wide spectrum of TTR stability, with rates of TTR disassembly ranging over 200-fold differences, can be achieved between WT and the two disease-associated variants of TTR studied here. Given that most patients carrying FAP-associated TTR mutations are heterozygous and may secrete heterotetramers with different stabilities, we speculate that an even greater range of stabilities may exist. The different magnitude of the stabilization offered by apo- and holoRBP between variants of TTR may imply that holoRBP is necessary to retard the disassembly of TTR, particularly for the L55P variant for which apoRBP offers only a marginal degree of stabilization. In the light of the observation that FAP patients typically display lower serum RBP concentrations when compared with normal controls (*32*), we can speculate that the a lack of holoRBP could lead to significant levels of free TTR tetramer which are prone to disassemble and advance the molecular pathology of SSA or FAP.

METHODS

Materials. Recombinant WT, L55P, and V30 M TTR (both ^{12}C–^{14}N and ^{13}C–^{15}N enriched forms) of TTR were expressed and purified as described previously (*28*). Isotopically labeled TTR was expressed in minimal medium with [^{15}N] ammonium chloride and [^{13}C] glucose (Spectra Stable Isotopes Inc.) as the sole nitrogen and carbon sources, respectively. The fidelity of recombinant TTR was confirmed by nanoESI-MS. Human retinol-binding protein (RBP), all-trans retinol, all-trans retinoic acid, 9-cis retinoic acid, all-trans retinal, all-trans retinyl acetate, L-thyroxine, ammonium acetate, and dimethyl sulfoxide (DMSO) were purchased from Sigma.

Subunit Exchange Reaction. In a typical subunit exchange reaction between [^{12}C–^{14}N] and [^{13}C–^{15}N] TTR, equal volumes of [^{12}C–^{14}N] and [^{13}C–^{15}N] TTR ($[TTR]_{Total}$ = 4.5 μM) were incubated at 37 °C for the times indicated in the absence of UV light. The reactions were sampled from an initial time point of ~2 min to 120 h. Triplicates of the subunit exchange reaction were performed for each experiment for WT V30 M and L55P TTR; [^{12}C–^{14}N] and [^{13}C–^{15}N] WT TTR; [^{12}C–^{14}N] and [^{13}C–^{15}N] V30 M TTR; and [^{12}C–^{14}N] and [^{13}C–^{15}N] L55P TTR. For subunit exchange of TTR in the presence of RBP, either apo- or holoRBP was added to an equimolar solution of [^{12}C–^{14}N] and [^{13}C–^{15}N] TTR immediately after mixing to a total concentration of 4.5 μM TTR and incubated at 37 °C. Typically, an aliquot of the solution (~1 μL) containing TTR was analyzed by a quadrupole time-of-flight (Q-ToF II) mass spectrometer (Waters, Manchester, UK) equipped with nanoflow ESI source. The instrument conditions were optimized to minimize complex disassembly during data acquisition (*33*) and to allow sufficient data to be collected over an appropriate time period. To induce gas-phase dissociation of the TTR:holoRBP complex into TTR and holoRBP within the mass spectrometer, the collision voltage that propels ions into the collision cell filled with argon was raised from 20 to 45 V. The intensity of peaks assigned to the 12+ and 13+ charge states of TTR composed of four [^{12}C–^{14}N] and [^{13}C–^{15}N] TTR monomers was summed and expressed as a fraction of the total intensity of all peaks assigned to the 12+ or 13+ charge states of TTR_4 in the spectrum. The relative abundances of the TTR homotetramer were plotted as a function of time.

In order to study the concentration dependence of holoRBP on the rate of TTR disassembly, holoRBP was added to an equimolar solution of [^{12}C–^{14}N] and [^{13}C–^{15}N] TTR to give a final concentration of holoRBP from 1.1 to 9 μM. In order to study the influence of retinoids (4.5 μM in 1% DMSO/H_2O) were added to an equimolar solution of TTR to give a final concentration of retinoids 4.5 μM.

For the analysis of TTR in complex with apoRBP or holoRBP, TTR stock solutions in 20 mM ammonium acetate were diluted to a final concentration of 4.5 μM of TTR. Apo- or holoRBP was added to the TTR to give a final concentration of 1.1, 2.3, 3.4, 4.5, or 9.0 μM of RBP. The binding of z-retinoic acid and thyroxine to TTR was studied by incubating 4.5 μM of TTR with molar excess of thyroxine and retinoic acid (3 and 4, respectively).

Kinetic Modeling. A kinetic model describing the subunit exchange process was developed as outlined before (*20*). The disassembly of the tetramer was allowed to occur *via* two pathways; disassembly across the dimer:dimer interface to form two dimers ($k_{d,dim}$) or disassembly of the tetramer to form a monomer and a trimer ($k_{d,mon}$). The reassociation of TTR monomer or dimer to form TTR tetramer was allowed to occur *via* two pathways, where a monomer associates with a trimer, or a dimer as-

sociates with another dimer. Numerical evaluation of the rate equations yields the concentration of each species (including the TTR tetramer and subunits/subcomplexes) that are involved in the kinetic schemes at a given time. The model software was produced in-house using Mathematica 6.0. Further details on the validation of kinetic model are provided in the Supplementary Method.

Acknowledgment: The authors gratefully acknowledge M. McCammon for assistance in protein expression and purification. E. Bromley and N. Barrera are acknowledged for helpful discussions about modeling. J. Benesch and M. McCammon are acknowledged for critical readings of the manuscript. S.J.H. acknowledges the Biotechnology and Biological Sciences Research Council, and C.V.R. is a Professor of the Royal Society.

Supporting Information Available: This material is available free of charge *via* the Internet.

REFERENCES

1. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors, *FASEB J. 10*, 940–954.
2. Kanai, M., Raz, A., and Goodman, D. S. (1968) Retinol-binding protein: the transport protein for vitamin A in human plasma, *J. Clin. Invest. 47*, 2025–2044.
3. Zanotti, G., and Berni, R. (2004) Plasma retinol-binding protein: structure and interactions with retinol, retinoids, and transthyretin, *Vitam. Horm. 69*, 271–295.
4. Ronne, H., Ocklind, C., Wiman, K., Rask, L., Obrink, B., and Peterson, P. A. (1983) Ligand-dependent regulation of intracellular protein transport: effect of vitamin a on the secretion of the retinol-binding protein, *J. Cell Biol. 96*, 907–910.
5. Wyss, R., and Bucheli, F. (1997) Determination of endogenous levels of 13-cis-retinoic acid (isotretinoin), all-trans-retinoic acid (tretinoin) and their 4-oxo metabolites in human and animal plasma by high-performance liquid chromatography with automated column switching and ultraviolet detection, *J. Chromatogr., B: Biomed. Sci. Appl. 700*, 31–47.
6. Arnhold, T., Tzimas, G., Wittfoht, W., Plonait, S., and Nau, H. (1996) Identification of 9-cis-retinoic acid, 9,13-di-cis-retinoic acid, and 14-hydroxy-4,14-retro-retinol in human plasma after liver consumption, *Life Sci. 59*, 169–177.
7. Quadro, L., Blaner, W. S., Salchow, D. J., Vogel, S., Piantedosi, R., Gouras, P., Freeman, S., Cosma, M. P., Colantuoni, V., and Gottesman, M. E. (1999) Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein, *EMBO J. 18*, 4633–4644.
8. Cogan, U., Kopelman, M., Mokady, S., and Shinitzky, M. (1976) Binding affinities of retinol and related compounds to retinol binding proteins, *Eur. J. Biochem. 65*, 71–78.
9. Blomhoff, R., Green, M. H., Green, J. B., Berg, T., and Norum, K. R. (1991) Vitamin A metabolism: new perspectives on absorption, transport, and storage, *Physiol. Rev. 71*, 951–990.
10. Zanotti, G., Berni, R., and Monaco, H. L. (1993) Crystal structure of liganded and unliganded forms of bovine plasma retinol-binding protein, *J. Biol. Chem. 268*, 10728–10738.
11. Sporn, M. B., Roberts, A. B., Goodman, D. S. (1984) *The Retinoids*, Vol. 2, Academic Press, New York.
12. Goodman, D. S. (1980) Plasma retinol-binding protein, *Ann. N.Y. Acad. Sci. 348*, 378–390.
13. McCammon, M. G., Scott, D. J., Keetch, C. A., Greene, L. H., Purkey, H. E., Petrassi, H. M., Kelly, J. W., and Robinson, C. V. (2002) Screening transthyretin amyloid fibril inhibitors: Characterization of novel multiprotein, multiligand complexes by mass spectrometry, *Structure 10*, 851–863.
14. Blake, C. C., Geisow, M. J., Oatley, S. J., Rerat, B., and Rerat, C. (1978) Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1.8 A, *J. Mol. Biol. 121*, 339–356.
15. Colon, W., and Kelly, J. W. (1992) Partial denaturation of transthyretin is sufficient for amyloid fibril formation in vitro, *Biochemistry 31*, 8654–8660.
16. Lai, Z., Colon, W., and Kelly, J. W. (1996) The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid, *Biochemistry 35*, 6470–6482.
17. Quintas, A., Vaz, D. C., Cardoso, I., Saraiva, M. J., and Brito, R. M. (2001) Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants, *J. Biol. Chem. 276*, 27207–27213.
18. Jacobson, D. R., Reveille, J. D., and Buxbaum, J. N. (1991) Frequency and genetic background of the position 122 (Val-Ile) variant transthyretin gene in the black population, *Am. J. Hum. Genet. 49*, 192–198.
19. Schneider, F., Hammarstrom, P., and Kelly, J. W. (2001) Transthyretin slowly exchanges subunits under physiological conditions: A convenient chromatographic method to study subunit exchange in oligomeric proteins, *Protein Sci. 10*, 1606–1613.
20. Keetch, C. A., Bromley, E. H., McCammon, M. G., Wang, N., Christodoulou, J., and Robinson, C. V. (2005) L55P transthyretin accelerates subunit exchange and leads to rapid formation of hybrid tetramers, *J. Biol. Chem. 280*, 41667–41674.
21. White, J. T., and Kelly, J. W. (2001) Support for the multigenic hypothesis of amyloidosis: the binding stoichiometry of retinol-binding protein, vitamin A, and thyroid hormone influences transthyretin amyloidogenicity in vitro, *Proc. Natl. Acad. Sci. U.S.A. 98*, 13019–13024.
22. Monaco, H. L., Rizzi, M., and Coda, A. (1995) Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein, *Science 268*, 1039–1041.
23. Painter, A. J., Jaya, N., Basha, E., Vierling, E., Robinson, C. V., and Benesch, J. L. (2008) Real-time monitoring of protein complexes reveals their quaternary organization and dynamics, *Chem. Biol. 15*, 246–253.
24. Rose, R. J., Verger, D., Daviter, T., Remaut, H., Paci, E., Waksman, G., Ashcroft, A. E., and Radford, S. E. (2008) Unraveling the molecular basis of subunit specificity in P pilus assembly by mass spectrometry, *Proc. Natl. Acad. Sci. U.S.A. 105*, 12873–12878.
25. Natan, E., Hirschberg, D., Morgner, N., Robinson, C. V., and Fersht, A. R. (2009) Ultraslow oligomerization equilibria of p53 and its implications, *Proc. Natl. Acad. Sci. U.S.A. 106*, 14327–14332.
26. McKay, A. R., Ruotolo, B. T., Ilag, L. L., and Robinson, C. V. (2006) Mass measurements of increased accuracy resolve heterogeneous populations of intact ribosomes, *J. Am. Chem. Soc. 128*, 11433–11442.
27. Wiseman, R. L., Johnson, S. M., Kelker, M. S., Foss, T., Wilson, I. A., and Kelly, J. W. (2005) Kinetic stabilization of an oligomeric protein by a single ligand binding event, *J. Am. Chem. Soc. 127*, 5540–5551.
28. Lashuel, H. A., Lai, Z., and Kelly, J. W. (1998) Characterization of the transthyretin acid denaturation pathways by analytical ultracentrifugation: implications for wild-type, V30M, and L55P amyloid fibril formation, *Biochemistry 37*, 17851–17864.
29. Bernstein, R., Robbins, J., and Rall, J. (1970) Polymorphism of monkey thyroxin-binding prealbumin (TBPA): mode of inheritance and hybridization, *Endocrinology 86*, 383–390.
30. Raghu, P., and Sivakumar, B. (2004) Interactions amongst plasma retinol-binding protein, transthyretin and their ligands: implications in vitamin A homeostasis and transthyretin amyloidosis, *Biochim. Biophys. Acta 1703*, 1–9.

31. Noy, N., Slosberg, E., and Scarlata, S. (1992) Interactions of retinol with binding proteins: studies with retinol-binding protein and with transthyretin, *Biochemistry 31*, 11118–11124.
32. Shoji, S., and Nakagawa, S. (1988) Serum prealbumin and retinol-binding protein concentrations in Japanese-type familial amyloid polyneuropathy, *Eur. Neurol. 28*, 191–193.
33. Hernandez, H., and Robinson, C. V. (2007) Determining the stoichiometry and interactions of macromolecular assemblies from mass spectrometry, *Nat. Protoc. 2*, 715–726.