Anion Shielding of Electrostatic Repulsions in Transthyretin Modulates Stability and Amyloidosis: Insight into the Chaotrope Unfolding Dichotomy†

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ABSTRACT: The balance between stabilizing forces and the localized electrostatic repulsions destabilizing the transthyretin (TTR) tetramer is tunable via anion shielding. The two symmetrical anion interaction sites in TTR are comprised of residues Lys15 and Lys15′ from opposing subunits on the periphery of the two thyroxine binding sites. These ϵ-ammonium groups repel one another and destabilize the tetramer, unless an appropriate anion is present, which stabilizes the tetramer. Chaotrope denaturation of TTR exhibits unusual behavior in that urea appears to be a stronger denaturant than GdmCl (guanidinium chloride), even though GdmCl is typically twice as powerful as a denaturant. The shift in the midpoint of the urea denaturation curve to higher concentrations as well as the increase in the mole fraction of tetramer that is highly resistant to denaturation with increasing KCl concentration provides strong evidence that anion shielding stabilizes the TTR tetramer. A consequence of tetramer stabilization is folding hysteresis, because the high GdmCl concentrations required to denature the anion-stabilized tetramer do not allow refolding of the unfolded monomers. The formation of amyloid fibrils by TTR requires that its normal tetrameric structure dissociate to alternatively folded monomers, a process mediated by acidification (pH 5−4). This process is inhibited by Cl^- ions in a concentration-dependent fashion. Chloride ion may not be the relevant physiological TTR stability modulator, but it is the main focus of these studies explaining the hysteresis observed in the denaturation and refolding studies with GdmCl.

Several disorders are known in which normally soluble folded proteins are converted into cross-β-sheet deposits called amyloid fibrils, which appear to cause disease. In cases where fibrils are composed of intact proteins, including transthyretin (*1*, *2*), lysozyme (*3*), immunoglobulin light chains (*4*), and β2-microglobulin (*5*, *6*), amyloid formation originates from partially folded intermediates. The population of partially folded intermediates can be increased by mutations and/or environmental stresses that facilitate partial denaturation (e.g., lysosomal pH-mediated denaturation). To elucidate the requirements of amyloid formation from this class of proteins, it is essential to understand the interactions that stabilize their native fold and how these can be disrupted.

Transthyretin (TTR)[1] is a 55 kDa homotetrameric protein present in plasma and cerebral spinal fluid that transports thyroxine and the retinol binding protein−vitamin A complex. Amyloidogenesis putatively causes the human amyloid diseases senile systemic amyloidosis (SSA) and familial amyloid polyneuropathy (FAP) involving deposition of the wild type or one of >80 single-site variants of TTR, respectively (*7*). The TTR tetramer dissociates to an alternatively folded monomer to assemble into amyloid fibrils, as a result of either a denaturation stress, a discrete mutation, or both (*8*, *9*). Previous data suggest that transthyretin is an unusually stable protein under certain conditions. In fact, previously published GdmCl denaturation and reconstitution curves exhibit dramatic hysteresis (see Figure 3B), suggesting that significant kinetic barriers exist between the folded tetramer and the unfolded monomer (*10*). Herein, we evaluate some of the variables that contribute to tetramer stability and hence amyloidogenicity, demonstrating that Cl^- interactions with the tetramer explain a number of the perplexing observations that characterize transthyretin. Our data suggest that hydrophobic interactions contribute significantly to TTR tetramer stability, whereas localized electrostatic repulsions between two pairs of Lys15 and Lys15′ residues destabilize the tetrameric structure. It is evident from this work that anion shielding of these charge repulsions can modulate tetramer stability and amyloid fibril formation, although it is premature to make statements about physiological relevance because the relevant anion may not be Cl^-.

MATERIALS AND METHODS

Protein Purification. Recombinant TTR was expressed and purified as previously described (*2*), with an additional gel filtration step employing a Superdex 75 column (Pharmacia, Uppsala, Sweden). Site-directed mutagenesis affording the Lys15Ala TTR variant was performed using the QuickChange protocol from Stratagene (*11*).

Urea, GdmCl, and GdmSCN Denaturation Curves Derived from Trp Fluorescence. Transthyretin (0.10 or 0.010 mg/

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[1] Abbreviations: TTR, Transthyretin; TTR_{wt}, wild type transthyretin; M-TTR, F87M/L110M TTR variant; GdmCl, guanidinium chloride; GdmSCN, guanidinium thiocyanate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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mL) stability was studied as a function of added denaturant and salt in 50 mM phosphate buffer (pH 7.0) with 1 mM EDTA and 1 mM DTT added. The protein was incubated for 40 h (GdmCl) or 20 h (GdmSCN) to be certain that the data collected were not time-dependent. Employing freshly prepared urea solutions, we opted not to exceed a 96 h incubation to avoid excessive modification of the protein by cyanate (unpublished results). Denaturant solutions were checked by refractive index to verify the concentrations prepared by weight. All TTR Trp fluorescence spectra were recorded over the range of 310−410 nm using an ATF 105 Aviv spectrofluorometer equipped with a thermostated cell holder employing excitation at 295 nm. Native wild-type TTR exhibits a fluorescence maximum at 337−338 nm, whereas the unfolded protein shows maximum emission between 355 and 358 nm. Each subunit of TTR has two tryptophans (residues 41 and 79). We used the 355 nm:335 nm emission intensity ratio to follow denaturation by the exposure of the tryptophans as a function of solvent denaturing conditions. The ratio increased from 0.85 ± 0.02 under native conditions to 1.3 ± 0.08 upon denaturation. The spectrum of a solution of tryptophan in the same buffer displayed a ratio of 1.4.

Thermal Denaturation. Thermal denaturation of TTR (0.10 mg/mL) in 50 mM phosphate buffer (pH 7.0 and 25 °C) (0.1 M KCl, 1 mM EDTA, and 1 mM DTT) was evaluated by monitoring the far-UV CD signal at 215 nm in a 2 mm cell using an Aviv CD spectrometer equipped with a thermoelectric cell holder. The CD signal was recorded from 2 to 100 °C in 3 °C steps, equilibrating the protein at each preset temperature step for 3 min.

Salt-Dependent Fibril Formation under Mildly Acidic Conditions. Amyloidogenicity of TTR_{wt} and Lys15Ala TTR was evaluated by mixing 500 μL of a 0.40 mg/mL solution of TTR in 10 mM phosphate (pH 7.0) containing 1 mM EDTA, 1 mM DTT, and varying concentrations of KCl (in the range of 0−1.8 M) with 500 μL of 200 mM acetate buffer (pH 4.3) (giving a final pH of 4.4) containing 1 mM EDTA, having the desired concentration of KCl. The samples were briefly vortexed and incubated for 72 h at 37 °C. The amounts of fibrils formed were quantified by measuring the turbidity of the suspentions by their absorbance at 400 nm.

Refolding. Refolding of TTR_{wt} (0.10 mg/mL), previously unfolded by a 96 h incubation in 8 M urea (0.1 M KCl), at 4 °C was accomplished by 10-fold dilution to the desired urea concentration. The protein was reequilibrated for 24 h before spectroscopic measurements were taken. In the case of the GdmCl refolding experiments, both TTR_{wt} and the Lys15Ala TTR variant were unfolded in 6.9 M GdmCl for 40 h at 4 °C and refolded by 10-fold dilution to the desired GdmCl concentration and reequilibrated for 24 h at 25 °C prior to spectroscopic analysis. Refolding of GdmSCN-unfolded TTR_{wt} was also performed by a 10-fold dilution of protein and denaturant initially unfolded in 3.0 M GdmSCN for 24 h to the desired final concentration of GdmSCN, which was equlibrated for 24 h before analysis.

To verify that the refolded TTR_{wt} protein attained the native fold, 7.2 mg of protein was unfolded at 4 °C for 48 h in 6.5 M GdmCl buffered with 50 mM phosphate (pH 7.0) containing 100 mM KCl, 1 mM EDTA, and 1 mM DTT. The protein was refolded at 25 °C by dilution to a final GdmCl concentration of 0.2 M in the above-mentioned buffer and at a protein concentration of 0.05 mg/mL. The refolding reaction was allowed to proceed for 24 h. The protein was subsequently concentrated and dialyzed versus the phosphate buffer and purified by gel filtration chromatography (Superdex 75, Pharmacia). The reconstituted protein was therafter unfolded by GdmCl and urea as described in a previous section to be sure that the refolded TTR and virgin TTR exhibited identical denaturation curves.

Chemical Cross-Linking, SDS−PAGE, and Analytical Ultracentrifugation. Samples of TTR_{wt} (0.10 mg/mL) were incubated for 96 h in 6.0 M urea in the presence of 0, 0.5, and 1.5 M KCl in 50 mM phosphate buffer (pH 7.0) with 1 mM EDTA and DTT added. Glutaraldehyde (25%, 100 μL) was added to 1.0 mL of the TTR solution, and the cross-linking reaction was allowed to proceed for 4 min before the reaction was quenched by addition of 100 μL of $NaBH_4$ (7% in 0.1 M NaOH). The cross-linked protein samples were isolated on a pD10 column in phosphate buffer (Amersham Pharmacia) and were thereafter mixed with reducing SDS cocktail (5%). The samples were boiled for 5 min before loading them onto a SDS−PAGE gel. The protein bands were stained with Coomassie and quantified by use of the program Scion Image (Scion Corp.).

The Lys15Ala variant was chemically cross-linked as described above after incubation for 96 h in 0 and 6.0 M urea. The cross-linked protein (in phosphate buffer) was thereafter analyzed by analytical ultracentrifugation measurements (the viscosity of concentrated urea solutions precludes equilibrium ultracentrifuge analyses in 6 M urea as the approch to equilibrium is very slow). This was performed using a temperature-controlled Beckman XL-I analytical ultracentrifuge (equipped with an An60Ti rotor and photoelectric scanner). A 100 μL cross-linked TTR sample was inserted into a double-sector cell and analyzed at a rotor speed of 17 000 rpm. Superimposable duplicate scans 3 h apart indicate that equilibrium was reached (24 h). The data were analyzed using the ORIGIN software program from Beckman.

Reengineering TTR To Be Monomeric. In a separate publication (*12*), we describe the preparation of the Phe87Met/Leu110Met TTR variant (M-TTR) which adopts a normally folded (X-ray crystallograhy) nonamyloidogenic monomeric structure at pH 7.5 which can be transformed into amyloid fibrils by mild acidification (pH <5.5). The GdmCl hysteresis observed for denaturation and/or reconstitution and anion stabilization described herein for the TTR tetramer are not properties exhibited by M-TTR.

RESULTS AND DISCUSSION

Electrostatic Effects on TTR Stability. Urea unfolding of wild-type transthyretin (TTR_{wt}) in the absence of added salt [50 mM phosphate (pH 7.0 and 25 °C)] displays a single reversible transition (Figure 1A) with a midpoint of unfolding at 3.4 M ($urea_{1/2}$). Interestingly, Figure 3B demonstrates that the midpoint of the GdmCl denaturation curve (irreversible) for TTR_{wt} is 5.3 M ($GdmCl_{1/2}$). This is opposite to the expected trend, as GdmCl as a denaturant is typically twice as potent as urea (*13*). The unfolding curve of TTR_{wt} in the potent denaturant GdmSCN exhibits the expected midpoint ($GdmSCN_{1/2}$ = 1.06 M) (Figure 6), although this denaturant also exhibits hysteresis when comparing denaturation and

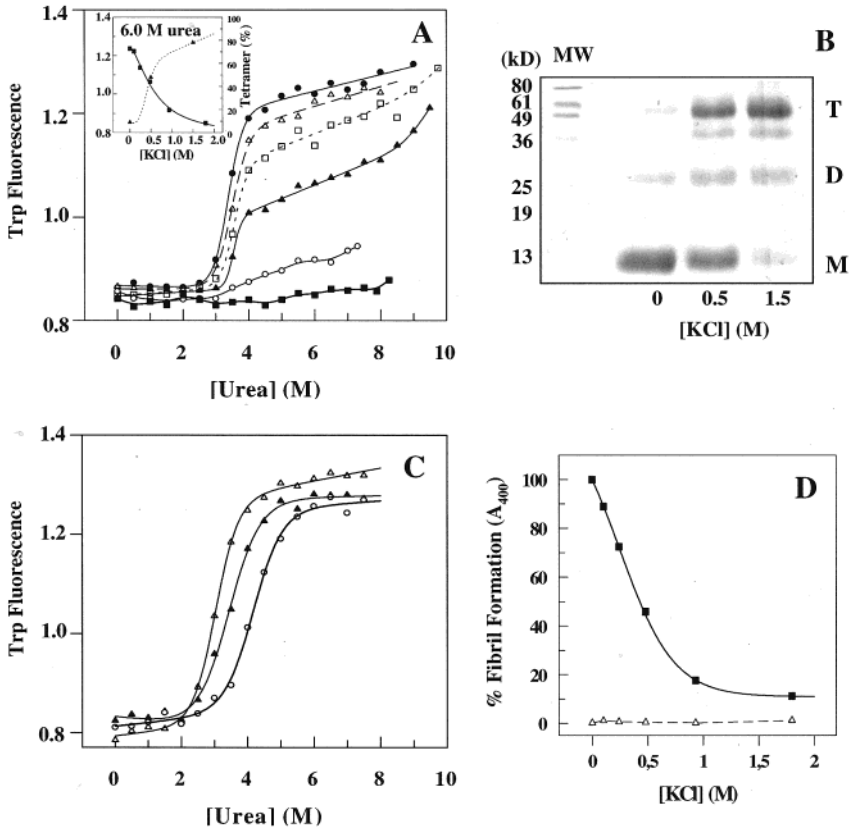

FIGURE 1: (A) Urea unfolding of 0.10 mg/mL TTR_{wt} (pH 7.0 and 25 °C) at various concentrations of KCl. The extent of unfolding was measured by recording the ratio of the tryptophan fluorescence intensity at 355 and 335 nm following excitation at 295 nm: 0.0 (●), 0.10 (△), 0.24 (□), 0.48 (▲), 0.93 (○), and 1.8 M added KCl (■). Lines are drawn to guide the eye. The inset shows the tryptophan fluorescence signal (■) together with the amount of tetramer (▲, dotted line) quantified by chemical cross-linking and SDS−PAGE (B) in 6.0 M urea as a function of KCl concentration. (B) SDS−PAGE gel of glutaraldehyde-cross-linked TTR_{wt} incubated for 96 h in 6.0 M urea in the presence of 0, 0.5, and 1.5 M KCl. Abbreviations: T, tetramer; D, dimer; M, monomer. The dimer band appears at constant intensity throughout the conditions used and could arise from incomplete cross-linking. (C) Urea denaturation of monomeric TTR (M-TTR) in the presence of varying concentrations of KCl, measured under the same conditions described for panel A: 0.10 (△), 0.50 (▲), and 1.0 M KCl (○). (D) Transthyretin (0.2 mg/mL) fibril formation analysis (turbidity recorded at 400 nm) at pH 4.4 as a function of KCl concentration for TTR_{wt} (■) and for the TTR Lys15Ala mutant (△).

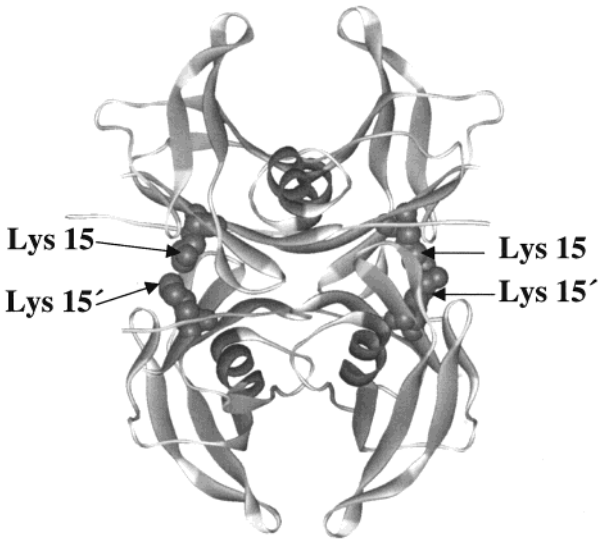

FIGURE 2: Ribbon diagram representation of the tetrameric TTR structure. The two pairs of Lys15−Lys15′ side chains are shown in ball-and-stick format.

reconstitution curves. The guanidinium-based reagents are ionic, whereas urea is neutral. Since GdmCl displays atypical impotence as a denaturant, it seems likely that the Cl^- counterion is stabilizing the TTR tetramer. To test the hypothesis that anion interactions change the stability of transthyretin, we evaluated the efficacy of urea denaturation as a function of KCl concentration [50 mM phosphate (pH 7.0 and 25 °C)]. It is apparent from Figure 1A and studies on a monomeric version of TTR (M-TTR; Figure 1C) that Cl^- stabilizes TTR in at least two ways. (i) Increasing Cl^- ion concentration increases the mole fraction of anion-stabilized tetrameric TTR that is highly resistant to denaturation (reflected by the amplitude change in Figure 1A). This is demonstrated by the chemical cross-linking experiments (Figure 1B). TTR_{wt} was cross-linked after incubation in 6.0 M urea for 96 h in the presence of 0, 0.5, and 1.5 M KCl. The amount of tetramer observed increases with Cl^- concentration [9% (at 0 M KCl) to 48% (at 0.5 M KCl) to 78% (at 1.5 M KCl)]. At high KCl concentrations (1.8 M), it is impossible to denature TTR_{wt} with urea on an experimentally accessible time scale, implying a strong stabilization by Cl^- (Figure 1A). One ramification of stabilizing TTR by anion interactions is that the kinetic barriers increase, making interconversions that are normally fast on an experimental time scale, such as tetramer dissociation, slower. A demonstration of this effect was recently published wherein we show that organic anion binding stabilizes the TTR tetramer to such an extent that it makes the barrier for TTR dissociation measured by subunit exchange unsurmountable

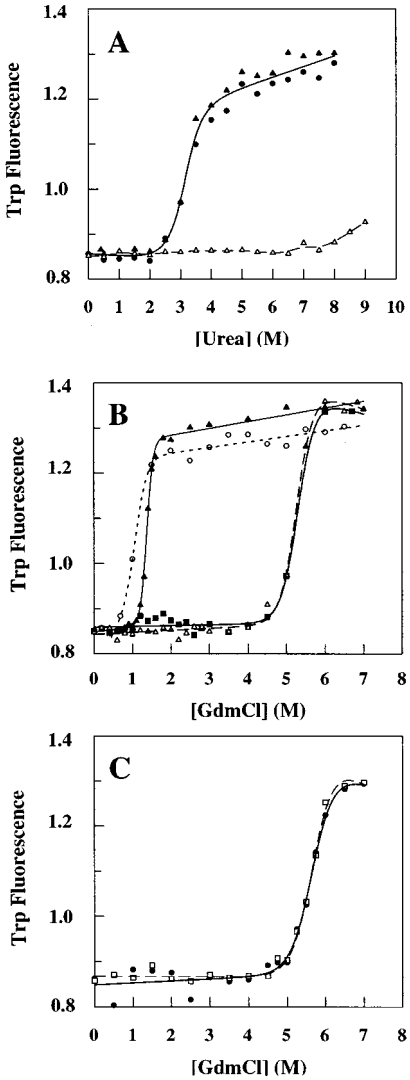

FIGURE 3: Comparison of the unfolding of TTR_{wt} and the Lys15Ala TTR variant at 25 °C in the presence of 100 mM KCl at a protein concentration of 0.10 mg/mL. The tryptophan fluorescence was recorded as described in Materials and Methods. (A) Urea unfolding curves of TTR_{wt} (●) and Lys15Ala (△), together with the reconstitution curve of TTR_{wt} (▲). (B) GdmCl unfolding curves of TTR_{wt} (■) and Lys15Ala (△) and reconstitution curves of TTR_{wt} (▲) and Lys15Ala (○). (C) Unfolding curve of native TTR_{wt} (●) and previously reconstituted TTR_{wt} (from 6.5 M GdmCl by dilution) (□), demonstrating that reconstitution does not lead to an alternatively folded state.

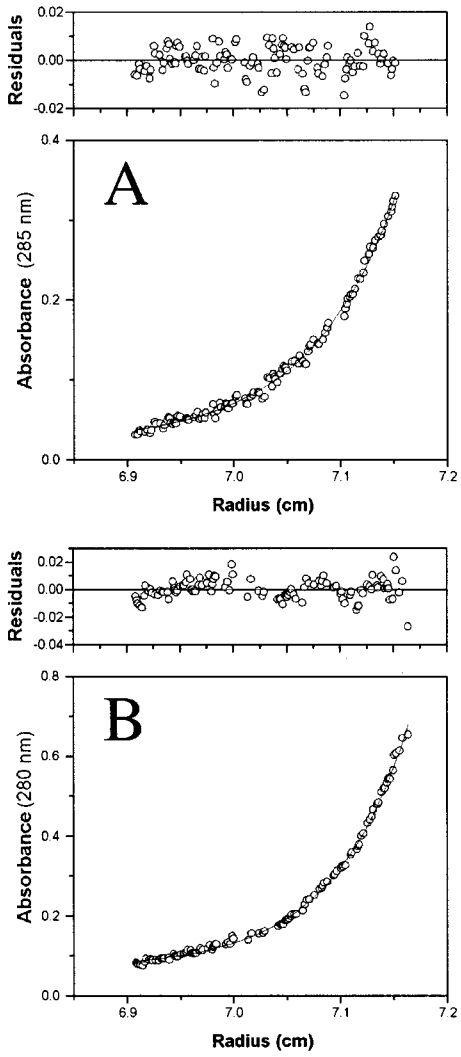

FIGURE 4: Sedimentation equilibrium analysis of Lys15Ala TTR incubated for 96 h in 0 (A) and 6.0 M (B) urea followed by chemical cross-linking (urea removed after cross-linking and before analysis). The lines represent curves fitted to a single-ideal species model providing MWs of (A) 49 599 ± 996 and (B) 56 570 ± 695. The insets show the distribution of random residuals.

under physiological conditions (*14*). The Cl^- binding curve shown in Figure 1A (inset) most likely underestimates the binding constant, as it is carried out in the presence of 6 M urea. Rationalizing all of the data in Figure 1A also requires knowledge about the tetramer dissociation rate as a function of Cl^- concentration, which will be further addressed by future kinetic studies. (ii) Increasing chloride ion concentration also modestly stabilizes the engineered monomeric version of TTR (M-TTR) toward urea denaturation (Figure 1C). Both M-TTR and TTR_{wt} [for the mole fraction accessible to denaturation (Figure 1A)] exhibit similar shifts in the midpoint of their denaturation curves as a function of Cl^- concentration, suggesting that in each case it is the monomer (resulting from dissociation) that is subject to denaturation. The observed chloride stabilization of the TTR monomer is typical for proteins and is not nearly as dramatic or as important as the tetramer stabilizing anion interactions with TTR that largely explain the data in Figure 1A.

Fluorescence-monitored urea denaturation of M-TTR shows that the fluorescence only decreases slightly (<10%) with increasing chloride concentrations (Figure 1C); hence, one cannot argue that the amplitude changes observed in Figure 1A result from anion quenching of the fluorescence or the like. Urea unfolding of TTR_{wt} appears to become biphasic at intermediate concentrations of KCl, because of the formation of urea resistant anion-stabilized tetramers.

We also explored the effect of varying the concentration of KCl on the propensity to form amyloid fibrils at pH 4.4 [the pH of maximum amyloidogenicity for TTR_{wt} (*2*)], where the net charge of TTR_{wt} would be approximately +9. Figure 1D illustrates that as in the case of Cl^--mediated stabilization of TTR against urea denaturation, we detect a concentration-dependent inhibititory effect on amyloid fibril formation over the concentration range of 0−1.8 M Cl^-, consistent with anion-mediated tetramer stabilization. Even though it is unclear whether Cl^- is the relevant physiologic anion, at its normal concentration (0.18 M) it reduces amyloidogenicity

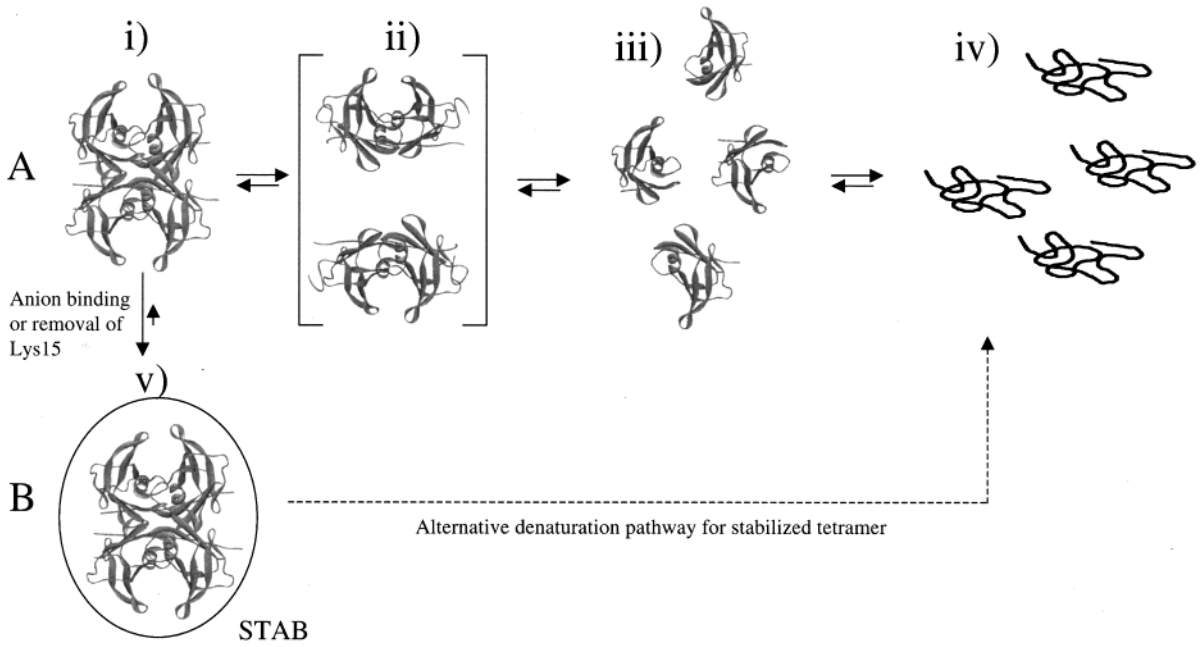

FIGURE 5: Model for folding and unfolding of TTR. In scheme A, the protein is unfolded in a sequential fashion with dissociation of the tetramer (i) via transient dimer formation (ii) which dissociates to folded monomers (iii) that subsequently unfold to an ensemble of unfolded chains (iv). Reconstitution is the reverse of this process. Scheme B illustrates unfolding of the anion-stabilized TTR tetramer directly (with anions shielding the two pairs of Lys15−Lys15′ residues), which requires a much higher denaturation stress. However, the initial steps of the reconstitution pathway are the same, since the anion stabilizing effect acts late in the pathway at the level of the quaternary structure. The observed folding hysteresis arises because the initial folding reaction is not possible under conditions required to denature the anion-stabilized tetramer.

by 20%, an effect that could have important biological implications.

All chaotrope stability measurements were performed at pH 7.0 where the protein has an overall negative charge of approximately −4 (pI of TTR is 5.5); hence, there is likely to be a specific anion interaction site(s) or region(s). Inspecting the structure for a possible anion binding site at pH 7.0 reveals a patch of positive charge composed of two Lys residues (Lys15 and Lys15′) projecting into the thyroid hormone binding site from neighboring subunits (Figure 2). The ϵ-ammonium group of a solvent-exposed lysine residue has a pK_a of ≈10, implying that these side chains are both positively charged at pH 7.0 and 4.4, unless an unexpected pK_a perturbation is involved. The Lys15 and Lys15′ ϵ-ammonium groups are known to make electrostatic interactions with the carboxylate group of thyroxine and numerous other small molecule anti-amyloid inhibitors that function via tetramer stabilization (*15*, *16*), based on crystallographic analysis. This interaction appears to be critical for inhibitor function.

In the absence of a negatively charged ligand or ion, it appears that the Lys15 and Lys15′ ϵ-ammonium groups repel each other. To test the hypotheses that these positively charged side chains form an anion interaction site and repel each other and destabilize the TTR tetramer in the absence of an appropriate anion, we replaced the solvent-exposed Lys15 side chain (which is not involved in any other side chain−side chain interactions) with an alanine residue. Remarkably, an assessment of amyloid formation derived from the Lys15Ala variant at pH 4.4 shows that this variant is completely nonamyloidogenic, even in the absence of salt (Figure 1D), consistent with a very stable tetramer vis-à-vis the lack of the Lys15 repulsions. Figure 3A shows the urea unfolding curves of TTR_{wt} and the Lys15Ala variant in the presence of 0.1 M KCl. That the Lys15Ala variant is highly resistant to urea-induced denaturation supports the hypothesis that the electrostatic repulsions between the Lys15 and Lys15′ C_2 symmetrical pairs of residues destabilize the TTR tetramer. In addition, this result provides circumstantial evidence that the intact tetramer may not be susceptible to urea denaturation (dissociation appears to be required for urea denaturation). Removal of these charges results in a protein that is dramatically stabilized as evidenced by the fact that the fluorescence reporting on denaturation does not even begin to change until a urea concentration of 7 M is reached, and then only modestly so. Chemical cross-linking of the Lys15Ala variant incubated in 6.0 M urea for 96 h followed by sedimentation equilibrium analysis after removal of the urea demonstrates that the protein retains its tetrameric structure (Figure 4). The data were fitted to a single-ideal species model with molecular weights of 49 599 ± 996 (0 M urea) and 56 570 ± 695 (6.0 M urea), both very close to the calculated molecular weight of the transthyretin tetramer (55 000).

As expected, the GdmCl unfolding curves of TTR_{wt} and the Lys15Ala mutant are virtually identical (Figure 3B). We interpret this to mean that anion interactions with the two Lys15−Lys15′ pairs stabilize the protein to a similar extent as achieved by removing the charges entirely (Lys15Ala). In the absence of the pair of Lys15−Lys15′ electrostatic repulsions (i.e., in the Lys15Ala variant or where Cl^- interactions dramatically reduce electrostatic repulsions in TTR_{wt}), 6 M GdmCl is required to completely unfold TTR. TTR_{wt} displays significant hysteresis in that the GdmCl concentration must be reduced to <2 M ($GdmCl_{1/2}$ = 1.4 M) to refold the unfolded monomers to a tetrameric structure (Figure 3B) (*10*). That the refolding curves of the Lys15Ala variant and TTR_{wt} are nearly identical (Figure 3B) implies

that the GdmCl concentration required to dissociate the tetramers is significantly higher than that which can support a folded structure in the monomers. These refolding curves of TTR reflect the "true" stability of the tertiary structure in the absence of anion stabilization of the tetrameric TTR (confirmed by studies on M-TTR (*12*)).

To verify that refolded TTR_{wt} was in fact native, we purified the reconstituted TTR_{wt} protein (denatured in 6.5 M GdmCl and refolded in 0.2 M GdmCl) and subjected the protein to a new round of GdmCl unfolding. The unfolding of the reconstituted protein can be superimposed on the unfolding of the virgin TTR, demonstrating that the hysteresis is not caused by formation of an alternatively folded structure during reconstitution (Figure 3C).

In other systems displaying folding hysteresis, the effect is kinetic in origin. For instance, the pro region of the α-lytic protease decreases the free energy of the transition state for folding (*17*) and the ground state of the native protein (*18*). In the absence of the pro region, the protein is meta stable and unfolds under native conditions, although very slowly because of a high kinetic barrier. In the case of TTR hysteresis, we invoke Cl^--mediated tetramer stabilization and a change in the unfolding mechanism to explain the hysteresis. The two unfolding and single folding schemes are presented in Figure 5 to further illustrate this point. These schemes are based on results of the study presented here together with previous published data and unpublished results. In scheme A, the unfolding of native TTR (i) proceeds in a sequential manner by dissociation of TTR to transient dimers (ii) that further dissociate to folded monomers (iii). The monomers can be urea denatured to an ensemble of disordered chains (iv). A period of 96 h is required to reach equilibrium in urea for these three steps. The folding reaction occurs in reverse order. The presence of high concentrations of anion or the Lys15Ala mutation (removing the Lys15−Lys15′ repulsions) results in stabilization of the tetramer (v), necessitating much more severe conditions for denaturation. The alternative unfolding pathway (scheme B) arises because TTR interacts with Cl^-, dramatically stabilizing the tetramer. One implication of this stabilization is that the already slow dissociation rate of the tetramer will further decrease if the anion stabilizes the tetrameric ground state more than the transition state for dissociation. Therefore, the Cl^--stabilized tetramer will only denature in powerful chaotropes by thermodynamic linkage of dissociation and unfolding, apparently not achievable in urea. It is reasonable to assume that the initial events on the reconstitution pathway are the same regardless of the influence of anions on the quaternary structure (scheme A) because the influence of Cl^- on monomeric TTR (M-TTR) is modest (Figure 1C). Because of anion interactions, the chaotrope concentration required to unfold the stabilized tetramer (scheme B) is high enough to prevent refolding of the monomers (scheme A) and hence cause the observed hysteresis.

Thus far, we have focused on Cl^- primarily to explain the hysteresis observed in GdmCl denaturation and reconstitution experiments. To ascertain whether chloride ions were the preferred anion for stabilization of TTR, other salts were evaluated. It is also important to demonstrate that the influence of the cation is minimal. Urea denaturation curves of TTR_{wt} in the presence of 0.93 M NaCl (data not shown) and KCl (Figure 1A) are identical, consistent with the importance of the anion and not the cation. The addition of 1.8 M KSCN stabilized TTR against urea denaturation (data not shown), although not to the same extent as 1.8 M KCl. Stabilization occurred despite the fact that SCN^- is the strongest "salting in" anion in the Hofmeister series. These results suggest that neutralizing the Lys15−Lys15′ repulsion can to some extent be accomplished by any anion. This result encouraged us to look for hysteresis effects in GdmSCN. GdmSCN denaturation and reconstitution of TTR also exhibits hysteresis (0 M KCl) (Figure 6), because of SCN^- interactions with tetrameric TTR. The midpoint of the unfolding transition is 1.06 M, while the midpoint for the refolding transition is 0.68 M. Because crystallization of TTR is accomplished using high concentrations of ammonium sulfate, we investigated the influence of ammonium sulfate on TTR denaturation. TTR is dramatically stabilized and could not be unfolded by urea in the presence of 0.95 M $(NH_4)_2SO_4$ (data not shown). Sulfate salts have also been shown to reduce the amount of fibril formation derived from TTR at low pH (*19*).

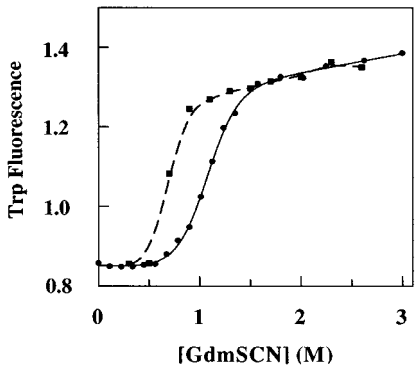

FIGURE 6: Unfolding and reconstitution hysteresis observed in GdmSCN. Unfolding (●) and refolding (■) curves of TTR_{wt} (0.10 mg/mL) in GdmSCN. The data were recorded using tryptophan fluorescence as described in Materials and Methods.

Properties of the Anion Interaction Site. In this report, we indirectly identify the anion interaction sites in TTR. The data strongly suggest the involvement of the Lys15−Lys15′ side chains at the dimer−dimer interface of TTR in this interaction. There is precedent for the direct structural characterization of anion binding sites in proteins by either X-ray crystallography or an NMR experiment (*20*, *21*). However, as in hemoglobin, for example, direct characterization of binding sites can be difficult. The established anion (chloride and organic phosphates) allosteric regulation of hemoglobin deserves special mention because it is analogous to the anion modulations of TTR stability established herein. Despite the fact that very low concentrations of Cl^- (10−200 mM) modulate allosteric transitions and Cl^- stabilization of the T state of hemoglobin, the search for a specific chloride binding site has been unfruitful (*22*), which is why we are cautious herein about referring to a TTR anion binding site. The oxygen affinity in hemoglobin is regulated by localized chloride ions that neutralize electrostatic repulsions formed by clusters of positively charged side chains in the central cavity between the subunits (*23*, *24*). Anions may stabilize TTR by a similar shielding mechanism.

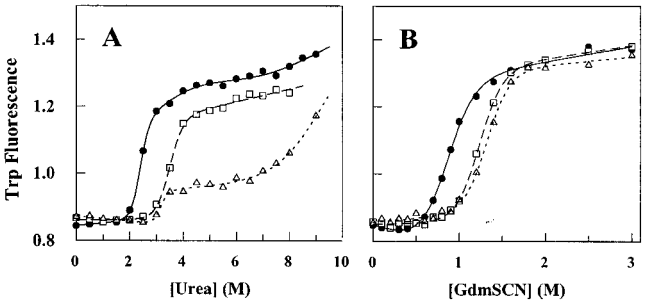

FIGURE 7: Chaotrope unfolding curves of TTR_{wt} at various temperatures in the presence of 100 mM KCl. The tryptophan fluorescence was recorded as described in Materials and Methods. (A) Urea unfolding curves, at 4 (●), 25 (□), and 37 °C (△). Lines are drawn to guide the eye. (B) GdmSCN unfolding curves, at 4 (●), 25 (□), and 37 °C (△).

Effect of Temperature on TTR Stability. TTR_{wt} is very stable toward thermal denaturation and does not commence unfolding at <80 °C (data not shown). This makes TTR one of the most thermostable plasma proteins (*25*). We investigated the influence of temperature on urea-induced unfolding of TTR_{wt}. Figure 7A shows the urea unfolding of TTR_{wt} at different temperatures, demonstrating that TTR is less stable toward chaotrope unfolding at lower temperatures. At increased temperatures, we found that TTR_{wt} cannot be completely unfolded by urea. A large plateu is evident in the urea denaturation curves at 37 °C, indicating the formation of intermediates that are either absent or barely populated at 4 °C. The lower stability at lower temperatures, where the hydrophobic effect is weaker, provides evidence that hydrohobic interactions play an important role in stabilizing tetrameric TTR. The temperature effect is also evident in GdmSCN unfolding measurements (Figure 7B). These hydrophobic interactions are evident in the crystal structure in that they contribute to both the edge-to-edge and face-to-face dimer interfaces stabilizing the quaternary structure (*26*). The importance of hydrophobic interactions is also consistent with the fact that TTR is less stable versus pressure-induced denaturation at lower temperatures (*27*). The decreased stability of the TTR tetramer at low temperatures is also evident in subunit exchange experiments, where exchange is significantly faster at 4 °C than at 37 °C (*14*).

Conclusions. Anion interactions with TTR shield the repulsive interactions between the Lys15−Lys15′ pairs at the two subunit interfaces stabilizing the tetramer, thus inhibiting amyloid fibril formation (by 20% at physiological Cl^- concentrations). The hysteresis encountered with GdmCl-induced denaturation and reconstitution, but not with urea, can be explained by the Cl^--induced stabilization of the TTR tetramer. The chaotrope concentration required to unfold the anion-stabilized TTR tetramer is high enough to preclude refolding of the monomeric structure, rationalizing the observed hysteresis.

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