

Molecular Transitions of Human Thyroxine-Binding Globulin

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The molecular transitions of thyroxine-binding globulin in guanidinium chloride solutions have been evaluated by circular dichroism, tryptophanyl fluorescence, and the polarization of tryptophanyl fluorescence at neutral pH. Below 2M guanidinium chloride, thyroxine-binding globulin undergoes a time-dependent irreversible transition which involves a randomization of almost half of the α -helical residues. At higher guanidinium chloride concentrations (2 to 5 M) thyroxine-binding globulin undergoes several further reversible molecular transitions. It appears that the molecular species obtained from serum is not the most stable form of this protein. The species present in 2 M guanidinium chloride, however, can be recovered from more highly unfolded forms of the protein.

Thyroxine-binding globulin (TBG),³ a specific transport protein for the thyroid hormones in human plasma, is a glycoprotein with a molecular weight of 54,000 and a single polypeptide chain (1, 2). Approximately 20% of the molecule is made up of carbohydrate, distributed in four oligosaccharide units of three types (3, 4). In neutral or alkaline aqueous solution, TBG has a compact, symmetric structure. Acidification below pH 5 results in a minor structural change with irreversible loss of hormone binding (5). The molecule is also easily denatured by mechanical agitation (6), by mild heating (7, 8) and during storage (6).

The first-order structural transition observed in acid is greatly accelerated in 1.8 M GdmCl solution (5). In the present work, we have investigated in more detail the effects of GdmCl on the molecular states of TBG. Our studies show an unusual kinetic

behavior pattern of TBG involving an irreversible transition at 2 M GdmCl concentration and a reversible transition among at least four different states between 2 and 5 M GdmCl solutions. This metastability of the native molecule raises important questions concerning the processing of TBG in the course of its biosynthesis or secretion.

MATERIALS AND METHODS

Materials. 5-Dimethylamino-1-naphthalenesulfonyl chloride was obtained from Aldrich. Guanidinium chloride, ultrapure grade, was purchased from Schwarz/Mann. Double glass-distilled water was used throughout. Chemicals not specifically mentioned were of reagent grade.

TBG preparation. TBG was purified from human plasma by a three-stage procedure employing affinity, anion exchange, and gel filtration chromatography (9, 2) and then dialyzed extensively against 0.1 M KCl, 0.05 M phosphate buffer, pH 7.5. The homogeneity and molecular characterization of this preparation have been reported previously (2). Most of the experiments reported here used a single preparation in which the residual T₄ content, determined by uv absorption (2), was 0.14 mol of T₄/mol of TBG. TBG concentration was determined by its absorption at 280 nm ($\epsilon_{1\text{cm}}^{280} = 6.2$) and corrected for the amount of T₄ present (2).

Circular dichroism. Circular dichroism in the far ultraviolet was measured with a Cary spectropolarimeter Model 60, equipped with a Pockel cell. The mean residue ellipticity of the peptide groups was calculated by

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³ Abbreviations used: TBG, thyroxine-binding globulin; DNS, dansyl, 5-dimethylamino-1-naphthalenesulfonyl; T₄, L-thyroxine; GdmCl, guanidinium chloride.

$$[\theta] = \frac{\theta \cdot 115}{10 c(\text{g/ml})l(\text{cm})},$$

where 115 is the assumed mean residue molecular weight of TBG. The CD data between 204 and 238 nm were analyzed by equations of Chen *et al.* (10) for the distribution of α -helical, β -structure, and unordered peptide groups. The program was constrained so that the sum of these three forms equaled 100%. An additional parameter which can be derived from the equations of Chen *et al.* (10) is the mean length of the α -helical segments.

Preparation of fluorescent conjugates. DNS-Cl in acetone was added to TBG (1.25 mg/ml in 0.1 M sodium bicarbonate buffer, pH 8.3) to give a molar ratio of added dye to protein of approximately 2:1. After 2 h in the dark at 4°C, conjugated DNS·TBG was separated from free dye by chromatography on Sephadex G-25. Employing absorbance at 340 nm, corrected for T_s absorption, and a molar extinction coefficient of 3360 for DNS (11), the number of moles of DNS bound per mole of protein was found to be 1.08.

Fluorescence intensity and polarization were measured with a Perkin-Elmer MPP3 spectrofluorometer, modified with a mechanical, automatic polarizing attachment (C. N. Wood Manufacturing, Newton, Pa.). Excitation and emission wavelengths were, respectively, 280 and 340 nm for tryptophanyl, and 340 and 490 nm for DNS. The temperature was 25°C. Polarization (P) is defined as $(I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$ where I is intensity, $G = I_{hv}/I_{hh}$, and the first and second subscripts, respectively, refer to the plane of polarization of the excitation and emission beams (v = vertical; h = horizontal).

Computer analysis. Least-squares fits were accomplished with an on-line modeling program, MLAB, developed at NIH (12). This program utilizes the Marquardt-Levenberg algorithm to perform nonlinear least-squares fits of experimental data to any arbitrary equation (13). Reported standard errors of fitted parameters correspond to approximately 1 SD.

The kinetic denaturation data were analyzed, in some cases, according to the equation (14, 15):

$$(Y - Y_{eq})/(Y_0 - Y_{eq}) = P_1 e^{-\theta_1 t} + P_2 e^{-\theta_2 t} \quad [1]$$

where Y , Y_0 , and Y_{eq} are the values of the observed variable at any time t , at time zero, and at equilibrium (infinite time), respectively. P_1 and P_2 are the amplitudes corresponding to the rate constants θ_1 and θ_2 . A complete discussion of this equation is given elsewhere (15).

The least-square fits presented in Table II were performed in two steps. First, the values of P_2 and θ_2 were determined on a truncated data set in which the initial rapid decay phase was deleted. In the second phase, several least-square fits to P_1 , P_2 , and θ_2 were performed with the entire decay curve and with the value of θ_1 fixed at various values. With values of θ_1

less than those presented in Table II it was found that the variance of the fit was significantly greater (as judged by an F statistic) than the variance for the initial determination of P_2 and θ_2 . The lower limit of θ_1 was consequently taken to be the value where the F statistic corresponded to a 65% probability (i.e., 1 SD). The value of P_1 was determined with a simultaneous fit to P_1 , P_2 , and θ_2 with θ_1 fixed at a value such that the values of P_2 and θ_2 were the same as determined in the first phase with the truncated data.

Two criteria were employed to test the ability of Eq. [1] to fit the experimental data. First, the variance between the fitted function and the experimental data must be approximately equal to the expected variance of the random experimental errors. Second, the deviations themselves must appear to be random. These criteria were applied qualitatively rather than quantitatively.

RESULTS

CD Spectra

The far-ultraviolet CD spectrum of native TBG at pH 8.3 is shown in Fig. 1. Using the spectra of Chen *et al.* (10), the percentage of α -helix, random coil, and β -structure were calculated (Table I). It is interesting to note that the mean length of α -helical segments required to fit the data is very short. The average value of 4.5 residues per α -helical segment is lower than any of the examples reported by Chen *et al.* (10) in the derivation of their spectra. In an earlier publication (5) we presented an analysis of CD spectra of native TBG assuming that the mean length of the α -helical regions was 10 residues. The present analysis gives a much better fit of the spectra. The addition of increasing amounts of GdmCl decreased the ellipticity until at 6 M the spectrum shows very little CD activity. Acidification to pH 3.4 in the absence of guanidine results in a decrease in the percentage of α -helix with a concomitant increase in the amount of β -structure. In 2 M GdmCl, there is an increase in the amount of random peptide groups, compared to the native molecule, which is at the expense of the α -helical residues. Acidification of a neutral solution of TBG in 2 M GdmCl to pH 4.0 had no effect within 18 h. Further addition of GdmCl decreases both the β -structure and α -helix residues. The nonzero values for α -helical and β -structure in 6 M GdmCl

(Table I) are probably due to a difference between the observed and model spectra of Chen *et al.* (10) but could indicate the presence of a small amount of residual structure.

Fluorescence and Fluorescence Polarization

Kinetic measurements showed that the conformational changes induced by GdmCl are complex and do not fit a two-state model. The first transition was seen at GdmCl concentrations below 2 M. As shown in Fig. 2, the rate of decrease of tryptophanyl fluorescence of TBG at pH 7.8 was dependent on the concentration of GdmCl. In 1 M GdmCl, the rate was too slow to measure. In 1.50 and 1.75 M the data fit a first order process with rate constants of 0.27 and $1.6 \times 10^{-4} \text{ s}^{-1}$, respectively. The polarization of tryptophanyl fluorescence of TBG also showed time effects corresponding in rate to the fluorescence transition (Fig. 3). The fluorescence and polarization at GdmCl concentrations of 2 M or less reached the same final values. Acidification to pH 2.8 of a 1.8 M GdmCl solution of TBG which had reached its final polarization value at pH 7.8 did not produce any additional effect on the polarization. Evidently the structural state of TBG in 1.8 M GdmCl is independent of pH, at least between 7.8 and 4.0 (5) (Table I) and probably between 7.8 and 2.8. This species represents a new stable form of the protein.

The structural state observed in 1.8 M GdmCl could not be reversed to the initial state observed in water at neutral pH. When

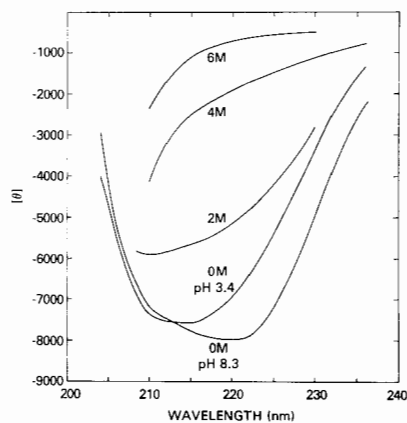


FIG. 1. Far-uv circular dichroism spectra of TBG at various GdmCl concentrations and pH. The pH values of the 2, 4, and 6 M solutions are given in Table I. The calculated percentages of α -helix, β -structure, and random coil for each of the curves are also given in Table I.

a solution of TBG in 2 M GdmCl at pH 7.8 was allowed to undergo its first order transition and was then diluted fourfold to 0.5 M GdmCl, neither the loss in fluorescence nor in polarization was recovered. A fluorometric titration curve with T_4 showed only 15% quenching and a nonlinear quenching curve. A similar titration of native TBG with T_4 in 0.5 M GdmCl gave linear quenching of about 60%, which was the same quenching curve as observed in the absence of GdmCl. When a TBG solution in 2 M GdmCl was dialyzed overnight against buffer to remove the GdmCl slowly, the fluorometric titration also showed very little quenching. It is evi-

TABLE I

CALCULATED PERCENTAGE α -HELIX, β -STRUCTURE, AND RANDOM STRUCTURE AND THE MEAN LENGTH OF α -HELICAL SEGMENTS FROM CD DATA IN FIG. 1

pH	[GdmCl] (M)	α -Helix		β -Structure (%)	Random coil (%)
		Percentage	Length ^a		
8.3	0	48	4.5	19	33
3.4	0	35	4.8	28	36
7.0 or 4.0	2	28	4.6	23	49
6.8 or 4.6	4	14	4.2	15	71
7.5 or 3	6	17	3.0	16	67

^a Average length of α -helical region expressed as the number of residues.

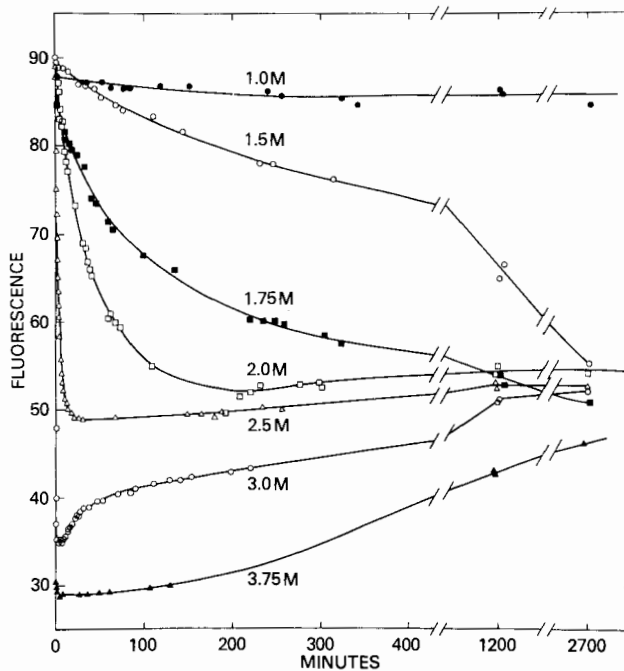


FIG. 2. The effect of GdmCl on tryptophanyl fluorescence of TBG (0.02 mg/ml) at pH 7.9 in 0.037 M PO_4 , 0.025 M Tris, 0.075 M KCl. The TBG was diluted from a buffer containing no GdmCl.

dent that the molecular transition of TBG in GdmCl solutions below 2 M results in an irreversible loss of the binding site for T_4 .

Further structural changes occur in TBG at neutral pH above 2 M GdmCl and lead to several new molecular species. The rate of formation of the initial new species increased very rapidly with GdmCl concentration above 2 M (Figs. 2, 3). After the initial rapid decrease in fluorescence and polarization in GdmCl solutions between 2.5 and 3.75 M both fluorescent parameters reached limiting values and then increased slowly to their equilibrium values. In 2.5 and 3.0 M GdmCl, the rate of decrease of fluorescence of TBG was slow enough to be followed and consequently minima were observed as the initial new species was transformed into another species with larger fluorescence. At 3.75 M GdmCl the rate of decrease of fluorescence was very rapid and only the subsequent rate of increase could be followed.

To determine whether this increase was due to association of unfolded TBG molecules or to intramolecular refolding, the rate of increase of polarization was measured

at two concentrations of TBG in 2.5, 3, and 3.75 M solutions of GdmCl. The polarization is the more sensitive parameter since it is a function of the relaxation time of the molecule. The results at 2.5 and 3.75 M are shown in Figs. 3 and 4, respectively. The rates were independent of three- to fourfold dif-

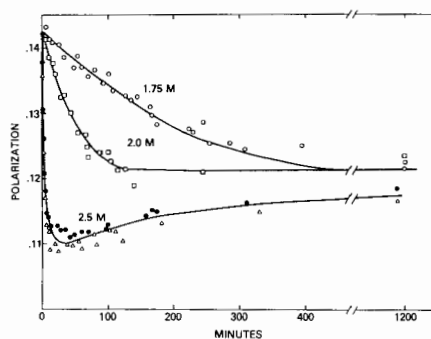


FIG. 3. The effect of GdmCl on tryptophanyl polarization of TBG (solvent as in Fig. 2). GdmCl, 1.75 and 2.00 M, contained 0.1 mg/ml TBG while the 2.5 M GdmCl experiment was performed at two concentrations: 0.075 mg/ml (Δ) and 0.30 mg/ml (\bullet). The TBG was diluted from a buffer containing no GdmCl.

ference in TBG concentration at all three levels of GdmCl and thus probably rule out an association reaction.

The DNS-labeled TBG behaved differently from unlabeled TBG at concentrations of GdmCl below 3 M and thereby offers another view of the properties of TBG. As in the case of tryptophanyl fluorescence in unconjugated TBG, DNS fluorescence and polarization did not change in 1 M GdmCl. Therefore the stability of TBG did not appear to be altered by the introduction of an average of one dansyl group per molecule of TBG. DNS fluorescence increased at a slow rate in 1.3 M GdmCl and at progressively faster rates at 1.8 and 2.7 M GdmCl (approximate half-times of 1, 60, and >600 min). At the latter two concentrations of GdmCl the fluorescence increase was over threefold. Also, in contrast to the tryptophanyl chromophore, the polarization of DNS fluorescence increased in 2 and 3 M GdmCl. When the rate of change in polarization was measured at two different DNS-TBG concentrations (0.1 and 0.3 mg/ml) in 3 M GdmCl an almost sixfold difference in the initial rate of change of polarization (approximate half-times of 115 and 20 min, respectively) was found. This increase in polarization was, therefore, at least in part due to an association of the unfolded form of TBG, and masks the decrease in polarization expected to result from the unfolding. Since the DNS fluores-

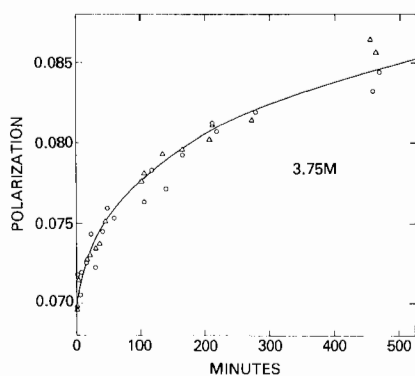


FIG. 4. The effect of TBG concentration on tryptophanyl polarization in 3.75 M GdmCl (solvent as in Fig. 2). TBG concentrations were 0.075 mg/ml (○) and 0.30 mg/ml (△). The TBG was diluted from a buffer containing no GdmCl.

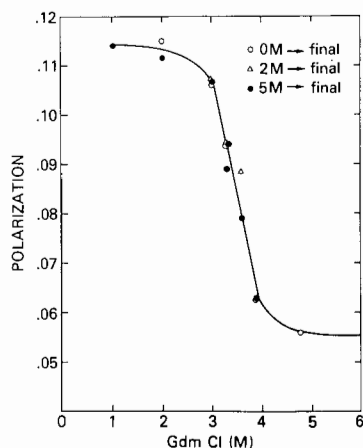


FIG. 5. The reversibility of the polarization of tryptophanyl fluorescence. The open circles and triangles indicate the equilibrium polarization values for the forward reaction starting with either 0 or 2 M GdmCl, respectively. The closed circles are for the reverse reaction starting with TBG in 5 M GdmCl.

cence change is much faster than the DNS polarization change in 2.7–3.0 M GdmCl solutions, the conformational transition precedes the self-association reaction.

In a separate experiment, the final polarization values were obtained as a function of GdmCl concentration by taking measurements only at 24-h intervals. Observations were made on TBG solutions which were initially in water, in 2 M GdmCl (end of reversible transition), or in 5 M GdmCl (final unfolded state). The transitions, which occur between 2 and 5 M GdmCl, involved a much larger change in polarization than between 0 and 2 M GdmCl. The data in Fig. 5 summarize the equilibrium polarization values and show that the unfolding was completely reversible between 2 and 5 M GdmCl. It should be noted that the polarization values of the reversal experiments only reached the value of the species obtained in 2 M GdmCl and not that observed in aqueous solution, i.e., the initial species. The changes in tryptophanyl fluorescence values (Fig. 2) showed that there is a rather large decrease in TBG fluorescence in the first transition (i.e., between 1 and 2 M GdmCl) but little fluorescence change at the higher GdmCl concentrations at equilibrium. Consequently, the large fall in polarization

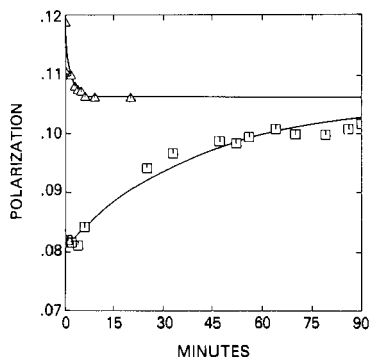


FIG. 6. Time course of tryptophanyl polarization for the forward reaction from 2 to 3 M GdmCl (Δ) and the reverse reaction from 4.8 to 3 M GdmCl (\square). The initial polarization values at 2 and 4.8 M were 0.119 and 0.058, respectively. Solvent as in Fig. 2. The lines are theoretical based on the constants given in Table II.

between 2 and 5 M GdmCl cannot result from an increase in lifetime and is therefore due to structural changes. This is confirmed by the major decrease in CD activity at higher GdmCl concentrations (Table I).

The time course of the approach to equilibrium at 3 M GdmCl for the forward and reverse directions is shown in Fig. 6 and Table II. The apparent first order rate constant for the forward transition from 2 to 3 M GdmCl is $0.0053 \pm 0.0010 \text{ s}^{-1}$. The corresponding apparent first order rate constant for the reverse reaction from 4.8 to 3 M GdmCl is $0.00041 \pm 0.00003 \text{ s}^{-1}$. In both cases, a significant portion of the decay occurred faster than we could measure.

The forward reaction from 2 M GdmCl to 3.3 and 3.6 M GdmCl is essentially complete within a few minutes and consequently we

were unable to measure the rate constants. The reverse reaction from 4.8 to 3.3 M GdmCl also has two phases, again one faster than could be measured by these techniques and a slower one with a rate constant of $0.00018 \pm 0.00002 \text{ s}^{-1}$. The nonidentity of the first order decay rates and initial rapid decays are typical of our data for the approach to each intermediate GdmCl concentration and indicate that the molecular changes which occur between 2 and 5 M GdmCl do not represent an equilibrium between only two species. If the transition were an equilibrium between two species, the apparent rate constant would be the sum of the microscopic forward and reverse rate constants (16) and would be independent of the initial conditions. It is therefore obvious that the denaturation reaction which occurs between 2 and 4.8 M GdmCl cannot be described as a simple two-state equilibrium.

DISCUSSION

In dilute GdmCl solutions (less than 2 M GdmCl) TBG undergoes a first order structural transition leading to a loss in hormone binding affinity and to modified molecular parameters. Both the secondary and tertiary structures are partially altered since there is a loss in the number of α -helical residues and significant changes in the fluorescence properties of the tryptophanyl residues. TBG in 2 M GdmCl appears to be a relatively stable molecular species since reducing the pH from neutrality to acid values has essentially no effect on the tryptophanyl fluorescence yield, polarization, or CD spectrum.

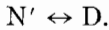
TABLE II
AMPLITUDES AND RATE CONSTANTS FOR GdmCl JUMPS^a

Concentration jump (M)	Relaxation component	p	θ (s ⁻¹)
2 to 3	1	0.63 ± 0.06	>0.19
	2	0.37 ± 0.06	$5.3 \pm 1.0 \times 10^{-3}$
4.8 to 3	1	0.46 ± 0.04	>0.022
	2	0.54 ± 0.04	$4.1 \pm 0.3 \times 10^{-4}$

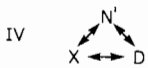
^a Analysis of the data presented in Fig. 6 according to Eq. [1].

Furthermore, TBG does not refold to the native state upon removal of GdmCl. It is therefore reasonable to conclude that the native state of TBG, i.e., the molecule processed from serum, may not represent its most stable form.

A further series of transitions occurs at GdmCl concentrations greater than 2 M which results in several more highly unfolded structures. It is easy to demonstrate that this is not a simple two state reaction between a structured state, N' (2 M GdmCl), and an unfolded state, D (5 M GdmCl), i.e.:



If this were the case, the data in Fig. 6 and Table II would have shown the same rate constant for the forward (from 2 to 3 M GdmCl) and reverse reactions (from 4.8 to 3 M GdmCl) (14). It can also be shown that the introduction of another species, X , can not completely explain the biphasic behavior of both curves (Fig. 6, Table II). Four possible three-state mechanisms can be formulated (15):

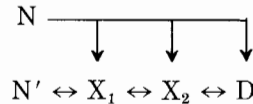


The time course of the approach to equilibrium for all four of these mechanisms can be described by Eq. [1] (14, 15), i.e., as the sum of two exponential decays. A consequence of the three-state model is that the rate constants for the two exponential decays, θ_1 and θ_2 , are independent of the initial conditions and are determined by the final equilibrium conditions (14, 15). Consequently, the values of θ_1 and θ_2 for the forward reaction (2 to 3 M GdmCl) must be identical to those for the reverse reaction (4.8 to 3 M GdmCl). Table II and Fig. 6 show that these reactions are biphasic, and the rate constants for the forward and reverse reactions are not the same. Therefore, the reversible equilibrium observed between 2 and 5 M GdmCl is an isomerization reaction involving more than three states or

some other mechanism. We have shown that the forward and reverse rates are independent of protein concentration and therefore molecular association can be excluded as an additional possibility for explaining the complex kinetics.

The transient minima observed by both fluorescence (Fig. 2) and polarization (Figs. 3 and 4) when native TBG is diluted into intermediate GdmCl concentrations is the result of the transient increase in the concentration of an intermediate between the native TBG, N , and the equilibrium state at 2 M GdmCl, N' . This intermediate has lower fluorescence and polarization values than the N' and is thus felt to be unfolded relative to the N' state. Below 2 M GdmCl the transient minima are not observed because the rate-limiting step in the transition between N and N' is the conversion of N to the intermediate, instead of the conversion of the intermediate to the N' state and thus the concentration of the intermediate never becomes significant. It should be noted that the first order transition of N to the intermediate has a large dependence on GdmCl as would be expected for a protein denaturation reaction.

The simplest mechanism for the unfolding of TBG in GdmCl can be depicted as:



It is not known if the intermediate in the transition from native TBG, N , to the N' state is X_1 , X_2 , D , or some other unfolded state. The four-state path $N' \leftrightarrow X_1 \leftrightarrow X_2 \leftrightarrow D$ is only one of many possible four-state mechanisms, and our experimental data are unable to distinguish between them. It should be noted that this scheme is the minimum mechanism and that more intermediates are possible.

Most single-chain globular proteins undergo reversible transitions induced by changes in pH, temperature, or by GdmCl. The apparent lack of reversibility of the first transition of TBG structure which takes place under rather mild conditions, i.e., dilute GdmCl at neutral pH or dilute acid, is difficult to explain. If, however, TBG

were synthesized as a larger molecule and then transformed by proteolysis to its form in serum, the failure to observe a reversal would be understandable. It is also possible that the addition of the carbohydrate chain modifies the stability of the secreted protein. Another possible explanation is that the solvent conditions where the TBG was initially folded, i.e., inside the cell, are such that the "native" state (no GdmCl) is the most stable. The change in solvent when the protein is released into the serum could then be sufficient to make the native state metastable and the state in 2 M GdmCl more stable. However, there is no direct evidence to support either hypothesis at the present time.

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