Structure and Stability of Human Thyroxine-binding Globulin*

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The secondary and tertiary structure of human plasma thyroxine-binding globulin (TBG) was investigated by circular dichroism and fluorescence properties. The relaxation time of TBG indicated that it is a compact, symmetric molecule. It was calculated from the far ultraviolet CD spectrum that about one-half of the peptide groups are equally distributed in α helical and β structures. In the near ultraviolet, the CD spectrum of TBG was modified when thyroxine was bound. TBG was stable at temperatures below 50° at pH 9 and below 35° at pH 10.5. Below pH 5 tryptophanyl fluorescence revealed a molecular transition which followed first order kinetics. The transition resulted in an irreversible loss of binding of the hormone. Acidification when thyroxine was bound. TBG was stable at temperatures near ultraviolet, the CD spectrum of TBG was modified for the aromatic chromophores which absorb in the near ultraviolet by

\[ \theta = 115 \times 10^2 \text{deg}(\text{cm} \cdot \text{dmol}^{-1}) \]

where \( \theta \) is the observed ellipticity, \( m \) is the molarity of TBG, \( \lambda \) is the pathlength. The mean residue ellipticity of the peptide groups which absorb in the far ultraviolet was calculated by

\[ [\theta] = \frac{\theta}{100} \frac{115}{\text{deg}(\text{cm} \cdot \text{dmol}^{-1})} \]

where 115 is the assumed mean residue molecular weight of TBG. The equations of Chen et al. (4) were used in a computer program to analyze the CD data between 204 and 238 nm for the distribution of α helical, β structure, and unordered peptide groups in the backbone chain. The program was constrained so that the sum of these three forms equaled 100%.

Ultraviolet Fluorescence—Fluorescence spectra and intensities were obtained with a Perkin-Elmer MPF3 spectrofluorometer at 25°. Excitation and emission wavelengths were 280 and 340 nm, respectively, for tryptophan and 340 and 490 nm for Dns intensity measurements.

Preparation of Fluorescent Conjugates—Microliter volumes of Dns-Cl dissolved in acetone were added to a solution of TBG (1.25 mg/ml) in 0.1 M sodium bicarbonate buffer, pH 8.3, at 4°. The molar ratio of added dye to protein was approximately 2:1. The reaction was allowed to proceed for 2 h in the dark at 4°, and the mixture was then chromatographed on a Sephadex G-25 column to separate the free from the conjugated dye. The number of moles of conjugated dye per mol of protein was determined by absorbance at 340 nm, after correcting for T, absorption, and using 4200 as the molar extinction coefficient of Dns (5).

Polarization of Fluorescence—The Perkin-Elmer MPF3 spectrofluorometer was modified with a mechanical, automatic polarizing attachment (C. N. Wood Manufacturing, Newton, Pa.) to measure polarization of fluorescence. Excitation and emission wavelengths were set, respectively, at 280 and 340 nm for tryptophan and at 340 and 490 nm for Dns. Polarization (P) is \( P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \) where \( I \) is intensity, \( G = I_{\parallel}/I_{\perp} \), and the first and second subscripts refer to the plane of polarization of the excitation and emission beams, respectively, \( (V = \text{vertical}; H = \text{horizontal}) \). The relaxation time (\( \tau_p \)) depends on the lifetime, temperature, and viscosity accord-

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The abbreviations used are: TBG, thyroxine-binding globulin; Dns, dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; GdmCl, guanidinium chloride; T, L-thyroxine.
Thyroxine-binding Globulin Structure

RESULTS

Structure of Native TBG

Secondary Structure—An estimate of the distribution of the peptide groups in TBG in ordered and unordered conformations was obtained from the far ultraviolet CD spectrum by using as models the CD spectra of proteins whose structures were known from x-ray analysis (4). The CD spectrum of TBG at pH 8.30 is shown in Fig. 1 and the computed distribution of peptide groups in \( \alpha \) helical, \( \beta \), and unordered structures is shown in Table I. About one-half of the peptide groups appear to be about equally distributed between \( \beta \) structure and \( \alpha \) helical segments. Analysis of the data using poly-L-lysine as a model (7) gave about 10% less \( \alpha \) helical and 10% more \( \beta \) structure, and the same percentage of unordered peptide groups. Acidification of TBG to pH 3.4 produced only a small change in the CD spectrum (Fig. 1). The calculated distribution evaluated by the method of Cheng et al. (4) suggests that a few of the \( \alpha \) helical residues are refolded into \( \beta \) structure in the transition in acid (Table I). Acidification to pH 1.0 had little further effect on the CD spectrum.

Tertiary Structure—Evidence for the organization of the aromatic chromophores in TBG can be obtained from their optical activity in the near ultraviolet. The CD spectrum contains peaks at 294 nm and between 265 to 255 nm, as well as a shoulder at about 270 nm (Fig. 2). These peaks can be identified with the tryptophanyl, phenylalanyl, and tyrosyl residues, respectively. The assignment of the tyrosyl band is uncertain since the CD spectrum is dominated by the other two chromophores and only part of the tyrosyl group is seen as a weak shoulder on the phenylalanyl band. The CD activity was enhanced at all wavelengths with addition of T, to give 1 mol/mol of TBG (Fig. 2). All the added T, was bound at the TBG concentration used in this experiment. When the T, concentration was further increased to 2 and 3 mol/mol of TBG there was no additional effect on the spectrum. The change in the CD spectrum of TBG due to the binding of 1 mol of T, showed two broad maxima near 285 and 325 nm (Fig. 2) corresponding to the absorption maxima of the first excited state of unionized and ionized T, respectively, and therefore represent the induced CD bands of both forms of T.

The increasing induced CD activity at wavelengths below 270 nm presumably arises from the second excited state of T.

Relaxation Time—The relaxation time of TBG was measured by fluorescence polarization after covalent labeling with Dns. The lifetime of the Dns fluorescence of labeled TBG at pH 7.3 and 24° was 15 ns. The lifetime at other temperatures was obtained from the temperature dependence of the fluorescence intensity assuming proportionality between lifetime and intensity. The dependence of \( 1/P \) on \( T/T' \) was linear only between 3 and 26° (Fig. 3). Above 26°, the polarization values diverged from the line extrapolated from lower temperatures. Above 50°, time-dependent increases in polarization were

\[
\frac{1}{P} = \frac{1}{P_0} - \frac{3\tau}{\rho P_0^2}
\]

where \( P_0 \) is the limiting value of \( P \) when \( T/T' \) is zero, and \( \tau \) is the fluorescence lifetime.

\[
\rho P^2 = \frac{\text{intercept}}{\text{slope}} \left[ \frac{\eta}{T_{n,0}} \right]^{25}
\]

The relaxation time (\( \rho P \)) of a rigid, unhydrated sphere of the same mass may be calculated from \( \rho P = 3\eta V/RT \) where the molecular volume \( V = M \sigma^3 \).

Fluorescence Lifetime Measurements—Fluorescence lifetime of Dns-TBG was measured at 24°, pH 7.3, with a modified nanosecond fluorescence lifetime apparatus (TRW Inc., El Segundo, Calif.) as described by Chen (6). The lifetime at other temperatures was calculated from the temperature dependence of Dns fluorescence. The fluorescence intensity of Dns fell almost linearly by one-third between 3 and 40°.

Materials—1-Dimethylaminonaphthalene-5-sulfonyl chloride (Dns-Cl) was obtained from Aldrich. Guanidium chloride (GdmCl), ultrapure grade, was purchased from Schwarz/Mann. Chemicals not specifically mentioned were of reagent grade.

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observed. These deviations from linearity suggest that an unlabeled TBG is stable to 50° under these conditions (see below). The time effects observed above 50° represent the increase in rotational freedom of Dns occurs above 26° since equation. This gives a relaxation ratio ($\rho_1/\rho_0$) of 1.1 since the dependence of the low temperature points by the Perrin equation time ($\tau_0$) of 49 ns was calculated from the linear sphere of the same molecular volume as TBG is 43 ns. This of TBG fluorescence. As can be seen in Fig. 6, 60% of the 7.9 after the first order decay in fluorescence was complete.

Loss occurred when a pH 4.1 solution was neutralized to pH of TBG was returned to pH 7.9. No recovery of the fluorescence a single, new, stable species is produced in the acid transition.

The reversibility of the acid transition was evaluated by $T_4$ quenching. TBG (0.02 mg/ml) in 0.1 M KCl, 0.05 M phosphate, pH 7.10 and 6.15 solutions and in 0.1 M KCl, 0.05 M phosphate, 0.025 M acetate for pH 4.50 to 3.25 solutions, temperature, 23°C. The wavelengths of excitation and emission were 280 and 340 nm, respectively. The first order rate constants at pH 4.60, 4.00 and 3.65 were 0.047, 0.92, and $2.3 \times 10^{-3}$ s⁻¹, respectively.

Fig. 4. The effect of pH on the kinetics of tryptophanyl fluorescence quenching. TBG (0.02 mg/ml) in 0.1 M KCl, 0.05 M phosphate for pH 7.10 and 6.15 solutions and in 0.1 M KCl, 0.05 M phosphate, 0.025 M acetate for pH 4.50 to 3.25 solutions, temperature, 23°C. The wavelengths of excitation and emission were 280 and 340 nm, respectively.

Stability of TBG

Mild acidification resulted in irreversible changes in the molecular and hormone-binding properties of TBG. The emission peak of TBG at neutral pH is at 330 nm and was not altered by acidification. Lowering the pH to 6.15 (Fig. 4) or 5.10 resulted in small reductions in TBG fluorescence intensity, but no time-dependent changes were found. At pH 4.50, a very slow rate of quenching was observed which increased rapidly below pH 4.50. The rate of change in fluorescence followed first order kinetics and a 40% loss in fluorescence intensity was observed when compared to neutral pH.

Polarization of tryptophanyl fluorescence was constant above pH 5 but fell at lower pH values concomitantly with the first order process observed by fluorescence. After completion of the acid transition the polarization remained constant when the pH was reduced further to pH 2 (Fig. 5). Evidently a single, new, stable species is produced in the acid transition.

The reversibility of the acid transition was evaluated by fluorescence and $T_4$ binding experiments. Only 15% of the decrease in fluorescence was recovered when a pH 5.0 solution of TBG was returned to pH 7.9. No recovery of the fluorescence loss occurred when a pH 4.1 solution was neutralized to pH 7.9 after the first order decay in fluorescence was complete.

The recovery of $T_4$ binding was evaluated by $T_4$ quenching of TBG fluorescence. As can be seen in Fig. 6, 60% of the fluorescence intensity of native TBG was quenched at a molar ratio of $T_4$ to TBG of 1 (0.85 mol of added $T_4$, plus 0.19 mol of $T_4$ present in the TBG preparation). After neutralization of a pH 5.0 solution to pH 7.9, only 50% of the fluorescence intensity of TBG was quenched at a molar ratio of $T_4$ to TBG of 0.35 (Fig. 6). After the first order transition at pH 4.1, very little (10 to 15%) of the fluorescence was quenched by $T_4$. In this case, the pH 4.1 solution was dialyzed for 20 h at 4°C against the pH 7.9 buffer. It is evident that the molecular change produced at pH 5.0 is incompletely reversed and at pH 4.1 is essentially irreversible.

In the case of Dns-labeled TBG, the polarization of Dns was modified below pH 5 in accord with the instability observed with unlabeled TBC in the same pH region (Fig. 5). However, Dns polarization increased, in contrast to the decrease of tryptophanyl polarization. This can be attributed to self-association of Dns-TBG when it is unfolded by acid. In the absence of Dns labeling, no evidence of self-association of TBG was found. It appears that the altered form of Dns-TBG produced in acid is less soluble than that of the unlabelled TBG and self-association results.

Dns-TBG also behaves differently from unlabeled TBG in 2 and 3 M GdmCl solutions. Dns-TBG shows a concentration-dependent increase in polarization in 3 M GdmCl, whereas unlabeled TBG shows a concentration-independent decrease of tryptophanyl polarization (unpublished data).
I I I

FIG. 6. Titration of tryptophanyl fluorescence of TBG by T₄: ●, native TBG; ○, reversal from pH 5.0; △, reversal from pH 4.1. All titrations were performed at pH 7.9 in 0.075 M KCl, 0.025 M Tris, 0.037 M phosphate, temperature, 23°C. TBG concentration was between 0.01 and 0.02 mg/ml. The T₄ content of this TBG preparation was 0.14 mol of T₄/mol of TBG.

The quenching curves of both Dns and tryptophanyl fluorescence of Dns-TBG produced by T₄ are very similar (Fig. 7). This suggests that most, if not all, of the protein molecules were labeled with Dns. T₄ was more weakly bound to Dns-TBG than to unlabeled TBG since larger molar ratios of T₄ to protein were needed to quench the fluorescence. The quenching of Dns fluorescence by T₄ was accompanied by a decrease in polarization in the neutral pH range. The binding of 1 mol of T₄/mol of Dns-TBG reduced the polarization of Dns fluorescence from 0.190 to 0.158 between pH 8 and pH 6. This significant change cannot originate from an increase in lifetime since Dns fluorescence is strongly quenched.

The effect of pH on TBG stability in 1.8 M GdmCl is seen in Fig. 8. In this solvent, the time-dependent tryptophanyl fluorescence changes observed in water below pH 4.5 were evident at neutral pH and the rate accelerated with increasing acidity. The fluorescence intensity fell to the same value at pH values between 6.10 and 4.40. Assuming that the same final fluorescence intensity also occurs at pH 7.15 and 6.65, the data at pH 7.15, 6.65, and 6.10 fit first order kinetics.

The behavior of TBG in alkali was quite different from that in acid. Tryptophanyl polarization was invariant between neutral pH and pH 9.6, but increased somewhat at higher pH (Fig. 5). Tryptophanyl fluorescence was quenched almost 50% between pH 8.5 and 11.0 and the quenching was essentially reversible. In the absence of a configurational change, the quenching of tryptophanyl fluorescence would be due to energy transfer to tyrosyl residues which ionize in this pH zone. The increase in polarization above pH 9.6 could be explained by a decrease in tryptophanyl lifetime due to the energy transfer process.

The stability of TBG in alkali was investigated further with Dns-labeled TBG since energy transfer is not possible from Dns to the aromatic residues of TBG. The fluorescence of Dns should remain constant since no groups in the chromophore ionize between pH 8 and 12. The polarization of Dns fluorescence was constant between pH ~6.5 and 11.5 (Fig. 5), indicating that TBG was stable in this pH region.

The stability in alkali was also confirmed by thermal denaturation studies. Thermal quenching curves of tryptophanyl emission of TBG indicated structural stability to 50°C since the curves were linear between 6 and 50°C at pH 7.5 and 9.0. Above 50°C, tryptophanyl fluorescence fell abruptly at both pH values; at pH 10.5 the break occurred at 35°C. Similar thermal stability of TBG in whole serum, as assayed by T₄ binding, has been described (9). The stability of TBG in alkali was also evaluated by titration of TBG fluorescence with T₄ at 25°C. Very little difference was observed in the T₄ quenching curves at pH 7.3, 8.2, and 8.9.

DISCUSSION

Analysis of the CD spectrum of TBG in the peptide absorption region indicates that the structured segments of the polypeptide chain contain both α helical and β structures. The near ultraviolet CD spectrum reveals prominent bands due to tryptophan and phenylalanine.

TBG appears to be stable in dilute alkali. This is evident from the reversibility of tryptophanyl fluorescence changes, the invariance of Dns polarization, and tryptophanyl quenching by T₄. TBG is unstable, however, below pH ~5 where there is a first order structural transition which is not reversed by neutralization. The binding of T₄ is greatly decreased by this transition, but the small changes in ultraviolet CD...
activity and in tryptophanyl polarization indicate that only a relatively minor loss in α helical and tertiary structure has occurred. There seems to be a specific species formed in the acid transition since the polarization and CD spectrum remain constant with further acidification. In the case of Dns-TBG, the unfolding that occurs below pH ~5 results in self-association since the polarization increases significantly. Similar association occurs in dilute GdmCl solutions at neutral pH with Dns-TBG but not with unlabeled TBG. 2 Evidently the addition of 1 or 2 hydrophobic residues of Dns to TBG is enough to make the acid form less soluble than the unlabeled protein and self-association results. This is similar to Dns-labeled fibrinogen, which is also less soluble than the unlabeled protein (10).

The instability increases in 1.8 m GdmCl so that TBG is no longer stable at neutral pH. As in aqueous solution, the rate increases rapidly with acidity and by pH 5.1 the transition is complete in about 1 min.

The observed susceptibility of TBG to irreversible denaturation is in keeping with findings by other investigators. Korcek and Tabachnik (11) observed that the capacity of TBG to bind T₄ was lost when dilute solutions of TBG were mechanically agitated. The loss of T₄ binding at temperatures above 52.5°C has been demonstrated (9, 12). Difficulties in retaining binding capacity have also been encountered during storage of purified TBG (11).

It is evident that the low pH employed in several earlier attempts to purify TBG (13, 14) would have resulted in serious losses in binding capacity. The irreversible transition in acid, which occurs at a pH higher than the apparent isoelectric point of TBG (pH ~4) (15), complicates the interpretation of recent studies attempting to demonstrate heterogeneity of TBG by isoelectric focusing (3). Anilinonapthalene sulfonic acid is bound in the T₄ site of TBG at neutral pH (16). The increase in fluorescence intensity of anilinonapthalene sulfonic acid below pH 6.4 and the red shift in emission maximum below pH 4.0 (16) presumably reflect the structural modifications observed by tryptophanyl fluorescence.

The affinity of TBG for both thyroxine and triiodothyronine (T₃) is maximal between pH 7 and 8 and decreases sharply at lower pH (11, 17). Since the pK of the phenolic group of T₃ is near 7, whereas that of T₄ is 8.5 in the unbound state (1, 18), this decrease in affinity of both hormones below pH 7 has been attributed to an alteration in the protein rather than in the ionized state of the phenolic group of T₃ (11, 17). The CD data in the near ultraviolet show that both the ionized and the un-ionized forms of T₃ are bound.

The reduced affinity of Dns-TBG for T₄ at neutral pH could result either from a structural change induced by Dns or from competition between Dns and T₄ for the same site. It has been shown that prealbumin, another T₄ binding protein of plasma, is affinity-labeled by Dns-Cl (19). Furthermore, anilinonapthalene sulfonic acid, a hydrophobic probe similar to Dns, is bound in the T₄ site of TBG (16, 17, 20). It seems likely, therefore, that TBG is also affinity-labeled by Dns-Cl and that the reduced affinity for T₄ is due to competition for the binding site. The large reduction in Dns fluorescence and polarization with T₄ binding could represent the displacement of the chromophoric moiety from a region involving the T₄ binding site to another region of the protein surface where it is less rigidly bound. However, a conformational origin of these fluorescence changes cannot be excluded at present.

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