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# Effects of Thyroxine Binding on the Stability, Conformation, and Fluorescence Properties of Thyroxine-Binding Globulin†

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ABSTRACT: The effects of thyroxine ($T_4$) on several molecular properties of human thyroxine-binding globulin (TBG) have been evaluated. Changes in the sedimentation constant and relaxation time indicate that TBG becomes more symmetric and compact when $T_4$ is bound. This modification in structure is associated with an increase in the stability of TBG to denaturation by either acid or guanidinium chloride. $T_4$ binding also produces changes in the emission and excitation spectra of TBG, reflecting different environments of the four tryptophanyl residues. $T_4$ preferentially quenches residues in a less polar environment. In addition, it alters the effect of the collisional quencher, acrylamide, so as to indicate a shift in the environment of some of the exposed tryptophanyl residues.

Thyroxine-binding globulin (TBG)[1] is the plasma protein found in higher vertebrates which has the greatest affinity for thyroid hormones and transports the majority of $T_4$ and $T_3$ in the circulation (Robbins et al., 1978). Human TBG is a single polypeptide chain ($M_r$ 54 000) (Gershengorn et al., 1977a) with about 20% of its weight in carbohydrates which are organized in four oligosaccharide chains (Zinn et al., 1978a,b). One mole of hormone is bound per mole of protein. Recent studies have shown that TBG loses its ability to bind hormones in dilute acid (pH <5) and in dilute guanidinium chloride (<2 M) solutions (Gershengorn et al., 1977b). In the present work, we have shown that $T_4$ enhances the stability

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[1] Abbreviations used: TBG, thyroxine-binding globulin; TBG·$T_4$, thyroxine-binding globulin complexed with $T_4$; GdmCl, guanidinium chloride; Dns, dansyl (5-dimethylamino-1-naphthalenesulfonyl); $T_4$, L-thyroxine; $T_3$, L-3,5,3′-triiodothyronine.

of TBG to both acid and guanidinium chloride inactivation and produces an alteration in structure which, presumably, is responsible for the increase in stability.

## Materials and Methods

### *Materials*

5-Dimethylamino-1-naphthalenesulfonyl chloride [dansyl chloride (DnsCl)] was obtained from Aldrich. Guanidinium chloride, ultrapure grade, was purchased from Schwarz/Mann. Chemicals not specifically mentioned were of reagent grade. Acrylamide was recrystallized from ethyl acetate.

TBG was purified from fresh, frozen human plasma (NIH Blood Bank) by $T_4$–agarose affinity chromatography, DEAE-Sephadex A-50 anion-exchange chromatography, and in some cases Sephadex G-150 gel filtration (Gershengorn et al., 1977a). The purified material was concentrated on a Diaflo 10-$\mu$m membrane and dialyzed extensively against 0.10 M Tris buffer, pH 8.2. A single band was observed by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis at pH 8.9. Most of the experiments reported here were carried out with a single preparation in which the $T_4$ content, determined by measuring the absorption at 325 nm (where only $T_4$ absorbed), was 0.2 mol of $T_4$/mol of TBG. In other experiments, TBG containing 0.28 mol of $T_4$/mol of TBG was used.

### *Methods*

*Preparation of Fluorescence Conjugates.* TBG was dansylated in the presence and in the absence of $T_4$. In the former, TBG (1 mg/mL) was dialyzed against 0.1 M sodium bicarbonate buffer, pH 8.3, at 4 °C and then mixed with the appropriate amount of $T_4$–Sepharose at 20 °C. Microliter volumes of dansyl chloride dissolved in acetone were added at 4 °C. The molar ratio of added dansyl chloride to protein was approximately 2:1. The reaction was allowed to proceed for 2 h in the dark at 4 °C, with occasional shaking (more frequently in the first 0.5 h of reaction). Then the $T_4$–Sepharose was transferred to a column and extensively washed with 0.10 M sodium bicarbonate, pH 8.3. The labeled TBG was eluted with 30 mM KOH solution at room temperature. The protein was then extensively dialyzed against 0.1 M KCl, 0.025 M Tris, and 0.037 M phosphate buffer, pH 8.1. When TBG was dansylated in the absence of $T_4$, the procedure was the same except that the $T_4$–Sepharose step was eliminated.

The number of moles of conjugated Dns (per mole of protein) in both preparations was 0.8, as determined by the absorbance at 340 nm after correcting for $T_4$ absorption, and using 3360 as the molar extinction coefficient of dansyl (Hartley & Massey, 1953).

*Ultraviolet Fluorescence.* Fluorescence spectra and intensity were obtained with a Perkin-Elmer MPF3 spectrofluorometer at 20 °C; excitation and emission wavelengths were 280 and 340 nm, respectively, for tryptophan and 340–490 nm for dansyl.

*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 340 and 490 nm for the dansyl chromophore. Polarization ($P$) is defined as $(I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$ where $I$ is the intensity, $G = I_{hv}/I_{hh}$, and the first and the second subscripts refer to the plane of polarization of the excitation and emission beams, respectively (v = vertical; h = horizontal). The polarization data were analyzed by the Perrin equation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right)$$

where $\tau$ is the lifetime and $\rho_h$ the harmonic mean relaxation time.

*Fluorescence Lifetime Measurement.* Fluorescence lifetimes of dansyl and tryptophan chromophores were measured at 24 °C, pH 8.0, with a modified nanosecond fluorescence lifetime apparatus (TRW, Inc., El Segundo, CA).

*Analytical Ultracentrifugation.* Ultracentrifugation was performed in double-sector cells at 22 °C in a Spinco Model E ultracentrifuge equipped with schlieren optics and a temperature control unit (RTIC). The partial specific volume of TBG (0.724) was calculated from the amino acid and carbohydrate composition (Gershengorn et al., 1977a) and their known partial specific volumes (Cohn & Edsall, 1943; Gibbons, 1972).

The effect of binding 1 mol of $T_4$ on the partial specific volume of TBG was calculated by $\bar{v} = \sum M_i\bar{v}_i/\sum M_i$ where $\bar{v}$ of $T_4$ was taken as that of iodine, i.e., 0.20. The $\bar{v}$ of TBG·$T_4$ is therefore 0.717 compared to 0.724 for TBG. The effect of this change in $\bar{v}$ and $M$ on $s^0_{20,w}$ of TBG·$T_4$ was obtained from

$$s^0_{20,w} = \frac{s^0_{20,w}}{M(1 - \bar{v}\rho)_{TBG}}(M + T_4)(1 - \bar{v}\rho)_{TBG\cdot T_4}$$

The frictional ratio was calculated from the sedimentation constant by

$$\frac{f}{f_0} = \frac{M(1 - \bar{v}\rho)}{s(6\rho\eta N)[3\bar{v}/(4\pi N)]^{1/3}}$$

where $f$ is the frictional coefficient and $f_0$ is the frictional coefficient for a sphere of the same molecular weight.

*Acrylamide Quenching Analysis.* Acrylamide fluorescence quenching data can be analyzed according to the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{sv}(Q)$$

where $F_0$ and $F$ are the tryptophan fluorescence intensities in the absence and presence of quencher ($Q$); $K_{sv}$ is the dynamic quenching constant.

## Results

(*I*) *Stability.* (*A*) *Acid Inactivation.* TBG is not stable in dilute acid (pH <5) and undergoes a minor molecular transition in which the affinity for $T_4$ is strongly reduced (Gershengorn et al., 1977b). Since the free energy of $T_4$ binding is very large (~14 kcal/mol), it seemed likely that $T_4$ could reduce the acid lability of TBG. Consequently, we have measured the rates of the acid transition of TBG and TBG·$T_4$ at three pH values. At pH 4.4, the fluorescence of TBG·$T_4$ remained constant whereas that of TBG decreased with a half-life of about 25 min (Figure 1). The smaller emission intensity of TBG·$T_4$ compared to that of TBG is due to the quenching of tryptophanyl emission by $T_4$ (Gershengorn et al., 1977b). At pH 3.5 and 3.1, the fluorescence of TBG·$T_4$ increased while that of TBG decreased, and the two approached or equaled each other at the end of the transition. No difference was observed in the rate of change of fluorescence between TBG and TBG·$T_4$ at either pH 3.5 or 3.1. The increase in intensity of TBG·$T_4$ presumably resulted from the loss of quenching by $T_4$. $T_4$ was probably released from its binding site and therefore had no influence on the final fluorescence. Thus, there appears to be a significant increase in TBG stability to acid when $T_4$ is bound.

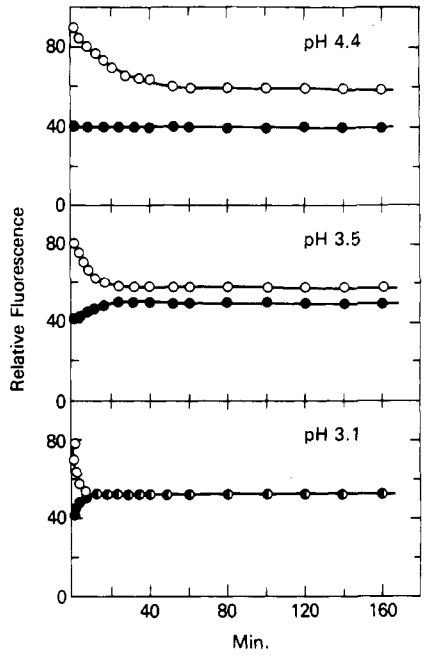


FIGURE 1: Effect of pH on the rate of change of the tryptophanyl fluorescence of TBG (○) and TBG·$T_4$ (●) (0.04 mg/mL) in 0.10 M KCl, 0.050 M phosphate, and 0.025 M acetate.

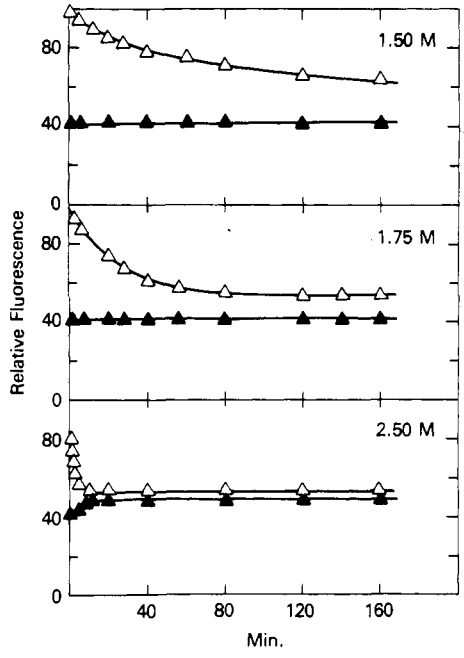


FIGURE 2: Effect of GdmCl concentration on the rate of change of the tryptophanyl fluorescence of TBG (△) and TBG·$T_4$ (▲) (0.04 mg/mL) in 0.037 M phosphate, 0.025 M Tris, and 0.075 M KCl, pH 8.10.

(*B*) *GdmCl Inactivation.* The pH range of the acid transition of TBG can be shifted to the neutral pH zone by adding low concentrations of GdmCl (Gershengorn et al., 1977b). As seen in Figure 2, increasing concentrations of GdmCl, between 1.5 and 2.5 M, resulted in increasing rates of fluorescence loss of TBG at pH 8.10. No time-dependent fluorescent change was observed with TBG·$T_4$ in 1.5 or 1.75 M GdmCl. A time-dependent increase in fluorescence was observed, however, in 2.5 M GdmCl, but the rate was much slower than with TBG. The binding of $T_4$ clearly has a significant effect in inhibiting the transition produced by GdmCl.

(*II*) *Conformational Effects.* In view of the enhanced stability of TBG·$T_4$ toward acid and GdmCl, it was of interest to determine whether the structure of TBG was modified by $T_4$. We have therefore evaluated the influence of $T_4$ on two conformational parameters (translational and rotational diffusion) as well as on its fluorescence spectrum.

Table I: Molecular and Fluorescence Quenching Parameters

| | TBG | TBG·$T_4$ |
|---|---|---|
| $s^0_{20,w} \times 10^{13}$ (S) | 4.00 | 4.63 |
| $f/f_0$ [a] | 1.23 | 1.11 |
| $\rho_{20}$ (ns) | 49 | 38 |
| $\rho/\rho_0$ | 1.40 | 1.07 |
| $K_{sv}^{275}$ ($M^{-1}$) | 33 | 36 |
| $K_{sv}^{290}$ ($M^{-1}$) | 11 | 8 |

[a] The molecular weight of the protein fraction of TBG was used to calculate $\rho_0$, i.e., 54 000 (0.80), since there is 20% carbohydrate present.

(*A*) *Sedimentation Velocity.* The concentration dependence of the sedimentation coefficients of TBG·$T_4$ and TBG was evaluated. Experiments were conducted on four pairs of solutions at identical concentrations of protein (between 2 and 4 mg/mL) in the same rotor. In all four pairs, the rate of sedimentation of TBG·$T_4$ was between 0.4 and 0.6 S less than that of TBG. A significant difference in extrapolated values of the sedimentation constants of TBG·$T_4$ and TBG (i.e., $s^0_{20,w}$, 4.63 and 4.00 S) was found. The value for TBG is very close to those reported by others for this constant (Giorgio & Tabachnik, 1968; Nilsson & Peterson, 1975).

An $s^0_{20,w}$ value of 4.13 S can be calculated from the value for TBG (i.e., 4.00 S), based on the change in $\bar{v}$ and $M$ expected when $T_4$ is bound (see Methods). The difference between the calculated and observed values for TBG·$T_4$ is outside of experimental error and indicates a decrease in the frictional coefficient of TBG with $T_4$ binding. The computed values for $f/f_0$ (see Methods) were 1.11 and 1.23 for TBG·$T_4$ and TBG, respectively (Table I).

(*B*) *Polarization of Fluorescence.* The rotational diffusion coefficient, i.e., relaxation time, of TBG was evaluated from the dependence of the polarization of dansyl fluorescence on the viscosity of the solution. TBG was covalently labeled with the dansyl chromophore in the absence and in the presence of $T_4$. Little or no difference was found in either dansyl fluorescence or polarization values between the two types of preparation. A lifetime of 17 ns was found at 20 °C for the dansyl group of labeled TBG, and this value did not change significantly when $T_4$ was bound.

The increase in the dansyl polarization of TBG·$T_4$ and TBG with addition of sucrose is plotted in Figure 3 according to the Perrin equation. The binding of $T_4$ resulted in a decrease in polarization. Relaxation times ($\rho_h$) of 38 and 49 ns at 20 °C were calculated from the data in Figure 4 for TBG·$T_4$ and TBG, respectively (Table I). The smaller relaxation time of TBG·$T_4$ suggests that binding $T_4$ results either in a decrease in the asymmetry of an equivalent ellipsoid of revolution, i.e., a decrease in the axial ratio, or in an increase in the rotational freedom of the segment(s) or domain(s) of TBG which contains the dansyl chromophores. On the basis of a dansyl lifetime of 15 ns, a relaxation time of 49 ns (25 °C) has been reported previously for TBG when the viscosity was varied by changing the temperature (Gershengorn et al., 1977b). These values are in agreement with those obtained in the present study.

The lifetimes of the tryptophanyl chromophores are normally too small to be useful for the application of the Perrin equation to proteins of the size of TBG. However, a significant decrease in the polarization of tryptophanyl fluorescence was observed with $T_4$ addition. In fact, the polarization titration

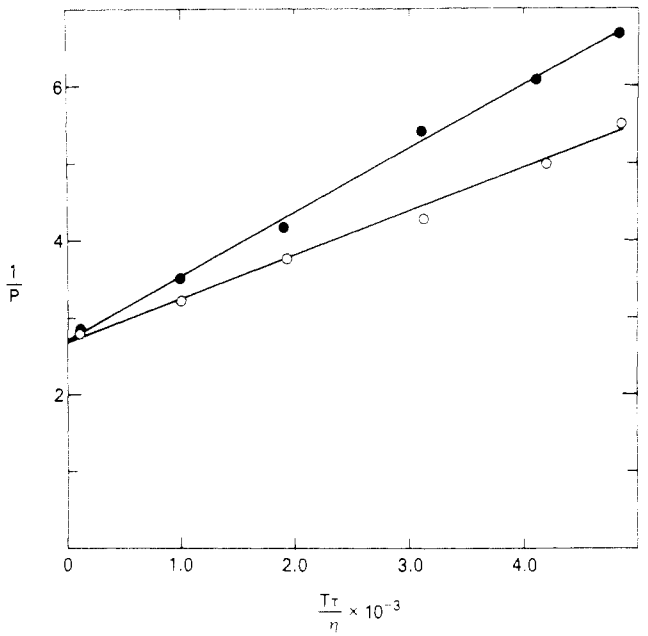


FIGURE 3: Perrin plots of the polarization of the Dns fluorescence of Dns·TBG (○) and Dns·TBG·$T_4$ (●) (0.04 mg/mL) in 0.037 M phosphate, 0.025 M tris, and 0.075 M KCl, pH 8.10. $T$ = 20 °C. The sucrose concentration varied from 5 to 60%. The wavelengths of excitation and emission were 340 and 490 nm, respectively. The lifetimes in sucrose solutions were obtained from the change in fluorescence with sucrose, assuming proportionality. The fluorescence of Dns·TBG decreased by 17% between 0 and 60% sucrose while that of Dns·TBG·$T_4$ decreased by 8%.

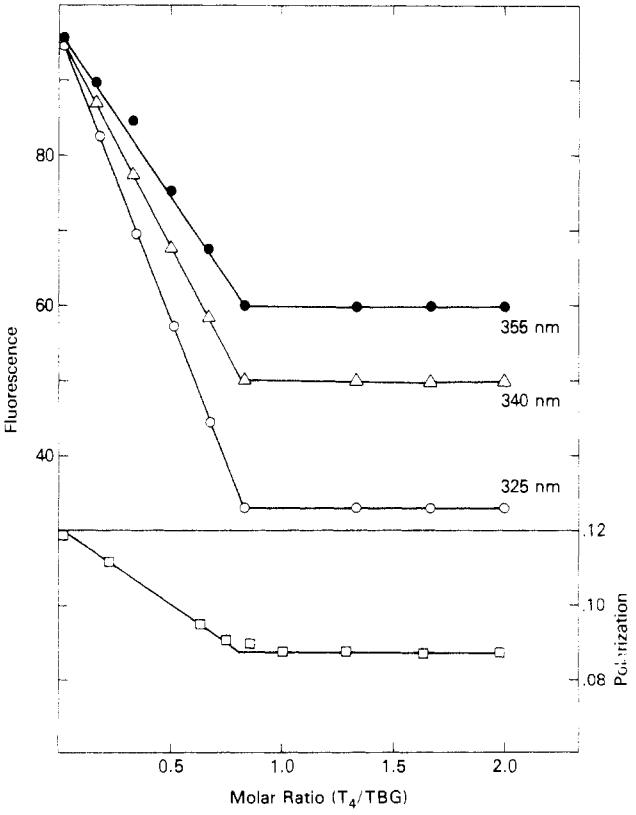


FIGURE 4: Titration of tryptophanyl fluorescence at three wavelengths and tryptophanyl polarization at 340 nm by $T_4$. The excitation wavelength was 280 nm. $T$ = 20 °C. Same solvent as in Figure 4.

curve also gives the stoichiometry of $T_4$ binding (Figure 4). A similar titration curve was obtained with $T_3$ although the decrease in polarization (and fluorescence) was somewhat smaller, i.e., 10% (not shown). The greater quenching by $T_4$ is expected if energy transfer is the major source of quenching of tryptophanyl fluorescence, since $T_4$ is more highly ionized at pH 8.10 and has a higher molar extinction coefficient than $T_3$ (Edelhoch, 1962). The decrease in polarization cannot be explained by an increase in the tryptophanyl lifetime since this was found to decrease from 3.6 to 2.4 ns, when $T_4$ was bound. Consequently, the tryptophanyl polarization data are in accord with the dansyl data and can be interpreted in the same way; i.e., the decrease in polarization is due either to an increase in the rotational flexibility of some of the domains containing tryptophanyl residues or to a decrease in the hydrodynamic volume, i.e., the frictional coefficient.

(*III*) *Tryptophanyl Heterogeneity*. The heterogeneity of tryptophanyl emission from multi-tryptophanyl-containing proteins has been demonstrated in numerous ways, including lifetime measurements (Shaklai et al., 1978; Gafni & Werber, 1979), variations in phosphorescent peaks (Purkey & Galley, 1970), quenching by collisional quenchers (Lehrer, 1967; Grinvald & Steinberg, 1974) and bound ligands (Secemski et al., 1972; Pollet & Edelhoch, 1973), by observing excitation spectra at two emitting wavelengths (Anderson et al., 1970; Kronman, 1976), and by circularly polarized emission (Vuk-Pavlović et al., 1979). We have used the uncharged collisional quencher acrylamide (Eftink & Ghiron, 1976) not only to show tryptophanyl heterogeneity but also to detect a conformational change produced by ligand binding.

(*A*) *$T_4$ Quenching*. The extent of tryptophanyl quenching by $T_4$ increased with decreasing emission wavelength, from 37% at 355 nm, to 47% at 340 nm, to 63% at 325 nm (Figure 4). This result implies that the emission spectrum of TBG is a composite of several overlapping spectra and that the individual emissions are quenched to different degrees by $T_4$. This effect could be directly observed by comparing the fluorescence spectra of TBG and TBG·$T_4$, since a shift in the emission peak from 330 to 335 nm was observed with $T_4$ binding (Figure 5A). The wavelength region of maximum quenching can be obtained by normalizing the peak of the $T_4$-quenched spectrum to the same peak intensity as the unquenched TBG spectrum. The spectrum obtained by subtracting the quenched spectrum from the unquenched spectrum had a peak at 325 nm (Figure 6A) and indicates that the tryptophanyl residues emitting at lower wavelengths were more strongly quenched than the others. These residues would be expected to reside in regions of the protein which are more excluded from contact with solvent molecules than residues situated near or on the surface. It is likely that the emission intensities of all the tryptophanyl residues are quenched, with those emitting at lower wavelengths being preferentially quenched. These data cannot be interpreted in terms of a conformational origin since energy transfer between tryptophan and thyroxine can occur over considerable distances and could explain the quenching results (Perlman et al., 1968).

(*B*) *Acrylamide Quenching*. Addition of acrylamide to a solution of TBG resulted in an emission spectrum with a peak shifted to 325 nm (Figure 5B). This result indicates that acrylamide quenches the more exposed tryptophanyl residues, i.e., those emitting at longer wavelengths, to a greater extent than the less exposed residues. The emission peak of the residues which are preferentially quenched can be resolved after normalization of the quenched with the unquenched spectra. The wavelength of strongest quenching is at 340 nm (Figure 6B).

When the above experiment was repeated with TBG·$T_4$, the acrylamide-quenched curve had a peak at 330 nm, i.e., 5 nm to the red of that observed with TBG (Figure 5C). After

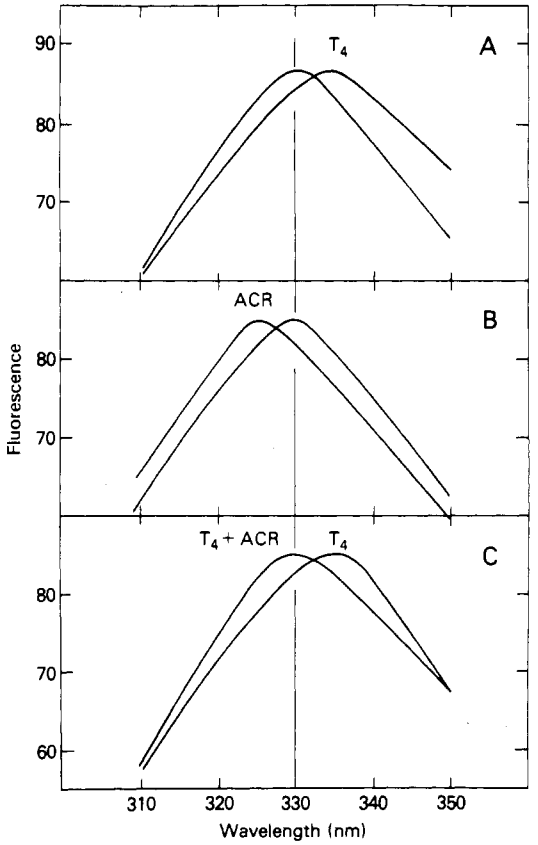


FIGURE 5: Effects of (A) $T_4$, (B) acrylamide, and (C) $T_4$ + acrylamide on the tryptophanyl emission spectra of TBG. All the spectra were normalized to the same peak intensity of TBG (0.04 mg/mL). The emission maximum of TBG is at 330 nm (vertical line). Same solvent as in Figure 4. The excitation wavelength was 280 nm.

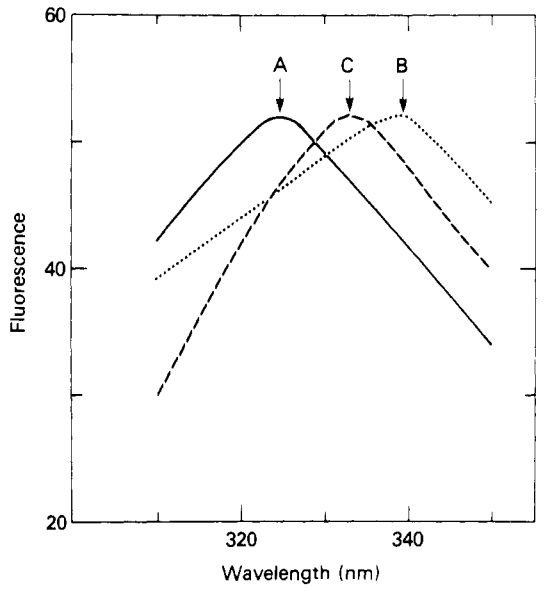


FIGURE 6: Difference in emission spectra after normalization, as shown in Figure 7, between (A) TBG·$T_4$ and TBG, (B) TBG·acrylamide and TBG, and (C) TBG·$T_4$·acrylamide and TBG·$T_4$.

normalization of the two spectra and subtraction, a peak at 333 nm was found (Figure 6C). This peak is blue shifted about 7 nm to the peak found in the absence of $T_4$ (Figure 6B). The displacement of the peak to the blue is contrary to expectations since $T_4$ preferentially quenches the groups at low wavelengths. This should increase the relative contribution of groups emitting at the longer wavelengths. If, in addition to highly exposed groups, the fluorescence of a less exposed group is

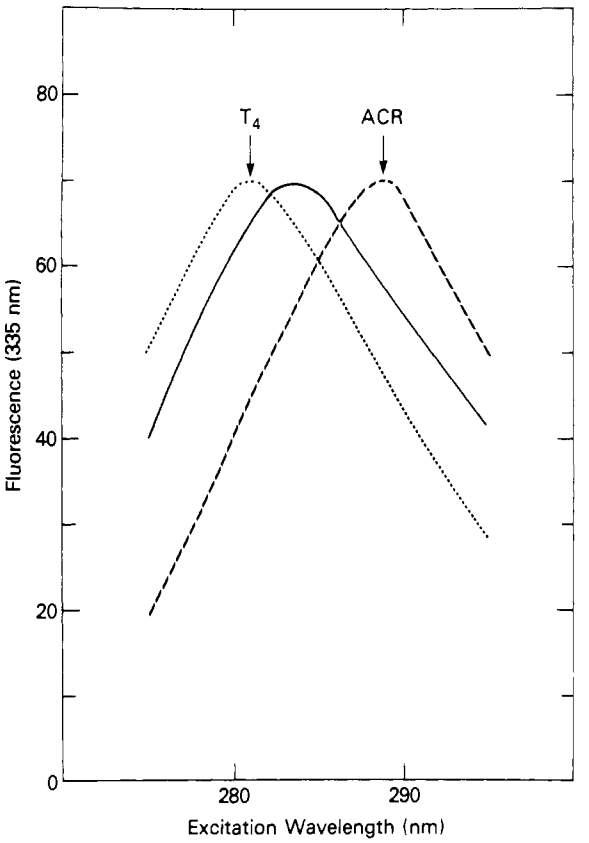


FIGURE 7: Tryptophanyl excitation spectra of TBG (—), TBG·$T_4$ (···), and TBG·acrylamide (---). Protein concentration was 0.04 mg/mL. Same solvent as in Figure 4. The wavelength of emission was 335 nm. All the spectra were normalized to the same peak intensity.

quenched by acrylamide as well as by $T_4$, then one would expect a red shift (after normalization and subtraction) with acrylamide in the presence of $T_4$. If less exposed groups are not quenched by acrylamide, then no shift would be expected, and the peak of the acrylamide-quenched groups (after normalization) would remain at 340 nm. The 7-nm blue shift (Figure 6C) can be interpreted, therefore, as resulting from a conformational change in which some of the more accessible tryptophanyl residues become less accessible after $T_4$ binding but are still quenchable by acrylamide.

(*C*) *Excitation Spectra.* The tryptophanyl residues which are more exposed to solvent molecules should absorb at shorter wavelengths compared to those which are less exposed, since organic compounds produce red shifts in absorption when added to aqueous solutions of model tryptophan compounds (Donovan, 1969). Consequently, if the emission of the less exposed residues is preferentially quenched by $T_4$, the emission of the more exposed residues should be more prominent, and the excitation spectrum should reflect this relationship. In accord with expectations, the excitation spectrum of TBG is blue shifted from 283.5 to 281 nm when it binds $T_4$ (Figure 7). Since acrylamide preferentially quenches the emission of the more exposed residues, the less exposed residues will emit more strongly, and the excitation peak will occur at longer wavelengths. The excitation curve of TBG is red shifted from 283.5 to 289 nm in the presence of acrylamide (Figure 7). An emission wavelength of 335 nm was used to obtain the excitation spectra since all the tryptophanyl residues will emit at this wavelength, albeit differently. Selection of different emission wavelengths would further increase the separation of the excitation spectra.

(*D*) *Stern–Volmer Analysis of Acrylamide Quenching.* The relative accessibility of the different tryptophanyl residues to

the solvent can also be ascertained by measuring the concentration dependence of acrylamide quenching at different exciting wavelengths. A $K_{sv}$ value of 33 $M^{-1}$ was found with TBG excited at 275 nm compared to a value of 11 $M^{-1}$ observed by excitation at 290 nm (Table I). The dependence is linear at 290 nm but slightly convex initially at 275 nm. This large difference in $K_{sv}$ values clearly indicates that there is an important selection of tryptophanyl residues at these two wavelengths. The smaller value of $K_{sv}$ observed at longer wavelengths indicates that residues which absorb at longer wavelengths (and emit at shorter wavelengths) are less easily quenched than those absorbing at shorter wavelengths. The residues absorbing at longer wavelengths are less in contact with the polar solvent molecules and would be expected to be less readily quenched by acrylamide. Smaller lifetime value(s) at 290 nm could, of course, account for some of the difference in $K_{sv}$ since $K_{sv} = k_3\tau$. The $K_{sv}$ value at 275 nm, i.e., 33 $M^{-1}$, is larger than those reported for *N*-acetyltryptophanamide, i.e., 17.5 $M^{-1}$, and for tryptophan in various proteins (Eftink & Ghiron, 1976). The unusually large value of 33 $M^{-1}$ presumably arises, at least in part, from noncollisional quenching by acrylamide, excluding a major difference in lifetimes. Static quenching has been shown to occur with acrylamide in a group of proteins containing only a single tryptophanyl residue. In this case, the Stern–Volmer plot is curved upward (Eftink & Ghiron, 1976). In the case of multitryptophanyl proteins, downward curvature was found. It is obviously very difficult to resolve the influence of static quenching from that of multiple lifetimes. It is evident that acrylamide quenching of TBG requires a noncollisional mechanism to explain the very large quenching constant observed.

The $K_{sv}$ value, when $T_4$ is bound, decreases from 11 to 8 $M^{-1}$ when excitation is at 290 nm, and increases slightly from 33 to 36 $M^{-1}$ at 275 nm (Table I). Thus, fewer groups remain accessible to acrylamide when the less exposed groups are preferentially excited at 290 nm. However, when the surface residues are preferentially excited at 275 nm, little change in $K_{sv}$ is observed.

## Discussion

The binding of low molecular weight ligands can enhance the stability of proteins. It has been well documented, for instance, that serum albumin can be preserved for blood transfusion purposes by adding small amounts of various organic solutes, such as long-chain fatty acids, acetyltryptophan, phenyl acetate, mandelic acid, and others (Boyer et al., 1946a,b). β-Lactoglobulin becomes more refractory to denaturation when it binds 2 mol of sodium dodecyl sulfate (Groves et al., 1951). A very large effect has been observed with avidin when it binds biotin which results in an increase in the thermal transition temperature from 85 to 132 °C (Donovan & Ross, 1973). The binding of these ligands may produce conformational changes which are responsible for the increase in resistance to denaturation.

TBG is a relatively unstable protein. A minor change in secondary and tertiary structure accompanies the irreversible transition of TBG when exposed to dilute acid or dilute GdmCl solutions. Major structural changes occur at higher concentrations of GdmCl which are, however, reversible (Johnson et al., 1980). Since TBG is fairly easily inactivated, it was thought that it might be stabilized by binding $T_4$, especially considering the very high affinity it has for this ligand, i.e., $\sim 10^{10}$. We have found that TBG·$T_4$ is more resistant than TBG to inactivation by either acid or GdmCl. Moreover, the enhanced stability can be attributed, at least in part, to a structural reorganization that occurs with $T_4$ binding.

The various methods employed clearly show changes with $T_4$ binding which can be interpreted in terms of a modification in molecular dimensions or in the degree of exposure of certain residues to the solvent. It should be evident that structural alterations necessarily result in variations in the degree of exposure of some residues.

The increase in $s^0_{20,w}$ and the decrease in $\rho_{20,w}$ and in tryptophanyl polarization can be interpreted in terms of TBG·$T_4$ being more symmetric than TBG. In fact, since the values of $f/f_0$ and $\rho/\rho_0$ for TBG·$T_4$ are only slightly greater than 1, the effective hydrodynamic shape of ligand-bound TBG cannot be far from that of a slightly hydrated, compact sphere. The experiments with acrylamide are in accord with the hydrodynamic data since they suggest that the tryptophanyl residues are less exposed in TBG·$T_4$ than in TBG. The reduced exposure implies a decrease in unfolding.

The heterogeneity of the environments of the tryptophanyl residues is clearly evident in the divergent effects of $T_4$ and acrylamide on both tryptophanyl emission and excitation. These two reagents do not interact exclusively with one or another tryptophanyl residue but only quench one type more strongly than the other. The preferential interactions monitored by the two quenching compounds might also be observed by lifetime measurements. However, with four tryptophanyl residues in TBG, the resolution of the four lifetimes may be very difficult, especially if they are not far from each other. Moreover, each lifetime would only be partly reduced by $T_4$ or acrylamide, and the resolution of individual lifetimes would remain difficult to accomplish.

## Acknowledgments

We thank Dr. Raymond Chen for the fluorescence lifetime measurements and Charlette Cureton for secretarial assistance.

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# Phosphorylation of Calcium Adenosinetriphosphatase by Inorganic Phosphate: Reversible Inhibition at High Magnesium Ion Concentrations†

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ABSTRACT: Magnesium stimulates phosphorylation of the calcium pump protein of the sarcoplasmic reticulum by inorganic phosphate, but the effect is reversed by high [$Mg^{2+}$]. This reversal is readily explained in terms of the generally accepted existence of two conformational states of the enzyme, $E_1$ and $E_2$. $E_2$ is the form of the enzyme that can be phosphorylated by $P_i$, and it has one binding site for $Mg^{2+}$. $E_1$ is the form of the enzyme that has two high-affinity $Ca^{2+}$ binding sites, and it is phosphorylated by ATP when $Ca^{2+}$ is bound. $Mg^{2+}$ can bind weakly to the two $Ca^{2+}$ sites and to a third site known to be present on $E_1$; this stabilizes $E_1$ at the expense of $E_2$ when [$Mg^{2+}$] is large. Stabilization of $E_1$ at pH 6.2 and 25 °C was found to be a highly cooperative function of [$Mg^{2+}$] and was not prevented by increasing [$P_i$]. The latter result requires the existence of a binding site for $P_i$ on $E_1$, with an affinity for $P_i$ comparable to that of $E_2$. Cooperativity with respect to [$Mg^{2+}$] requires that $E_2$ is the stable state of the enzyme in the absence of ligands, with an equilibrium constant [$E_2$]/[$E_1$] on the order of $10^3$ or higher at pH 6.2 and 25 °C.

The $Ca^{2+}$-ATPase of skeletal muscle sarcoplasmic reticulum can exist in two major conformational states, $E_1$ and $E_2$ (de Meis & Vianna, 1979). $E_1$ has two high-affinity sites for $Ca^{2+}$, a high-affinity site for ATP, and possibly a second site of lower affinity for ATP. When both ATP and $Ca^{2+}$ are bound, $E_1$ is converted to an ADP-sensitive phosphoenzyme, $Ca_2E_1$–P. $E_2$ binds one $Mg^{2+}$ and one inorganic orthophosphate ($P_i$)[1] ion and, when both are bound, can be converted to an ADP-insensitive phosphoenzyme, $MgE_2$–P.

In a previous paper (Martin & Tanford, 1981), we studied the thermodynamics of formation of $MgE_2$–P from unliganded enzyme ($E_1$ + $E_2$). At low $Mg^{2+}$ concentration (to about 10 mM), the extent of phosphorylation was found to obey the simple bireactant scheme shown in the right half of Figure 1, and our results and derived equilibrium constants were found to be in good agreement with the previous data of Punzengruber et al. (1978). At high [$Mg^{2+}$], however, the extent of phosphorylation was found to decrease, in qualitative agreement with earlier observations by Kanazawa (1975) and de Meis (1976). We suggested that the simplest way to account for this result is in terms of the stabilization of the $E_1$ form of the enzyme by high [$Mg^{2+}$]. The principal purpose of the present paper is to examine this possibility in more detail.

It is known that the formation of $MgE_2$–P is suppressed by the presence of even low levels of $Ca^{2+}$, owing to the stabilization of the $E_1$ state by the formation of $Ca_2E_1$. It is also known, however, that $Mg^{2+}$ can compete with weak affinity for the $Ca^{2+}$ binding sites (Yamada & Tonomura, 1972; Kalbitzer et al., 1978), and there is good evidence (Dupont, 1980; Kalbitzer et al., 1978) that there is a third binding site for divalent cations on $E_1$, which has a higher affinity for $Mg^{2+}$ than for $Ca^{2+}$. There is thus the possibility for formation of complexes between $Mg^{2+}$ and $E_1$ up to a level of $Mg_3E_1$ which can suppress $MgE_2$–P formation in the absence of $Ca^{2+}$ by the same thermodynamic principle by which low levels of added $Ca^{2+}$ lead to suppression. The equilibria involved in this mechanism are shown in the left half of Figure 1. Binding of $Mg^{2+}$ to $E_1$ is expressed in the figure in terms of the suc-

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‡ Recipient of a postdoctoral fellowship from the Muscular Dystrophy Association.
§ Chaim Weizmann postdoctoral fellow.

[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $P_i$, inorganic phosphate (ortho); Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-0151$01.25/0 