Interaction of Carbohydrate and Protein in Thyroxine Binding Globulin

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ABSTRACT: The fluorescence properties of human thyroxine binding globulin were evaluated during enzymatic deglycosylation by using both neuraminidase and a mixture of glycosidases. Three fluorescent chromophores, one intrinsic and two extrinsic, were monitored, and all showed changes in fluorescent parameters that have been interpreted in terms of a loss of interactions between the carbohydrate and amino acid residues during deglycosylation. The loss of carbohydrates also results in a decrease in stability of the protein to both acid and guanidinium chloride inactivation. Since deglycosylation decreases the frictional ratio of thyroxine binding globulin, it is concluded that, although sialic acid and other sugar residues are in contact with the protein surface, the hydrated carbohydrate chains protrude partially into the solvent.

Rapid advances have been made in recent years in our understanding of the chemical structure of the carbohydrate chains of glycoproteins, especially the asparagine-linked conjugates. Much of this development has come from biosynthetic studies (Snider & Robbins, 1981; Hubbard & Ivatt, 1981). Much more limited progress has been made, however, on the nature of the interactions between the carbohydrate and amino acid residues of glycoproteins and the biochemical consequences of these interactions. In some cases, the properties of the two structures appear to be distinct; e.g., the hormonal activity of human chorionic gonadotropin (hCG)[1] is lost with deglycosylation, whereas its membrane binding and antigenic activities, which apparently are confined to the apoprotein, remain intact (Kalyan & Bahl, 1983).

Modifications in protein stability and susceptibility to enzymic hydrolysis have been observed in some proteins after deglycosylation (Sairam & Manjunath, 1982) although many enzymes retain their specific activities after the loss of most of their carbohydrates (Wang & Hirs, 1977; Trimble & Maley, 1977; Chu et al., 1978). Differences have been reported in both the physical properties and enzyme activity of deglycosylated yeast external invertase after reversal from guanidinium hydrochloride denaturation (Chu et al., 1978). On the other hand, Wang & Hirs (1977) found no difference in the rates of renaturation between the reduced forms of native and deglycosylated RNase. In the case of the G protein of vesicular stomatitis virus, however, the high mannose asparagine-linked carbohydrates may play a significant role in preserving the properties of the nascent polypeptide chain during translational processing (Trimble & Maley, 1977; Gibson et al., 1981).

Carbohydrates can influence the properties of their protein conjugate in diverse ways. They can provide a steric shield to inhibit penetration by large and even small molecules, or modify the solubility of proteins since they are much more soluble than polypeptides. Under favorable conditions, they may be able to interact with specific residues in the protein, probably more easily with surface groups but, potentially, also with interior groups.

It is part of the dogma of the energetics of protein stability (Kauzmann, 1959; Nozaki & Tanford, 1971) that the carbohydrates, because of their high solubility in water, are preferentially accommodated outside the domain(s) of the protein and, therefore, do not play a direct role in influencing the higher levels of structure in the native protein, i.e., secondary, tertiary, and quaternary structure. This point of view is supported by thermodynamic considerations which indicate that hydrophobic and not hydrogen-bonding interactions provide the necessary free energy change to drive the linear nascent polypeptide into its native conformation (Kauzmann, 1959; Klotz & Farnham, 1968; Krescheck & Klotz, 1969). All the information for the proper folding of the chain is considered to be present in the sequence of amino acids (Haber & Anfinsen, 1962; Anfinsen, 1973).

There are relatively few studies with typical globular glycoproteins which directly evaluate the effect of the carbohydrate chains on the molecular properties of the protein in dilute aqueous solution. We have, therefore, tried to characterize an interaction in TBG between the amino acid and carbohydrate residues which affects some of the molecular parameters of the protein. TBG is a plasma protein containing 20% carbohydrate in 4 asparagine-linked, complex-type carbohydrates, with an average of 10 terminal sialic acid residues and 4 tryptophan residues (Zinn et al., 1978a,b; Cheng et al., 1979; Robbins & Edelhoch, 1985). In order to remove the carbohydrate residues of TBG, we have used two enzyme preparations: neuraminidase removes only the terminal sialic acid residues, while the mixed glycosidase preparation releases about 86% of the carbohydrates without much effect on the affinity for thyroxine (Cheng et al., 1979). We now report on a conformational change associated with deglycosylation of TBG as monitored by three fluorescent probes, one intrinsic and two extrinsic. We also show that deglycosylation decreases the stability of TBG.

Materials and Methods

Fresh, human plasma was obtained from the NIH Blood Bank. Thyroxine (T_4) was from Sigma. We are indebted to

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[1] Abbreviations: hCG, human chorionic gonadotropin; TBG, thyroxine binding globulin; dTBG, deglycosylated thyroxine binding globulin (missing 86% of the carbohydrates); T_4, thyroxine; Dns, dansyl [5-(dimethylamino)-1-naphthalenesulfonyl]; ANS, 8-anilino-1-naphthalenesulfonate; GdmCl, guanidinium chloride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

Dr. G. A. Ashwell (NIH) for the mixed glycosidase preparation from *Diplococcus pneumoniae*, type 1. Neuraminidase was from Sigma (*Clostridia perfringens*, type X, 125–150 units/mg), and α-mannosidase (T. Cornutus, 4.3 units/mg) was obtained from Miles Laboratories. 8-Anilino-1-naphthalenesulfonate (ANS) as the Mg salt was purchased from Eastman. 5-(Dimethylamino)-1-naphthalenesulfonyl chloride (dansyl chloride, DnsCl) was from Pierce.

The preparation of purified TBG and deglycosylated TBG has been described in earlier publications from this laboratory (Gershengorn et al., 1977a; Cheng et al., 1979; Grimaldi et al., 1982). It was shown by Cheng et al. (1979) that (1) 86% of the carbohydrates are removed by the mixed glycosidase preparation, (2) no new terminal groups were found in deglycosylated TBG after six steps of the Edman degradation, indicating that the mixed glycosidase preparation was free of proteolytic activity, (3) a single band was found for dTBG by SDS gel electrophoresis with a molecular weight of 43 700 which is very close to the calculated weight of 44 000, and (4) a similar molecular weight was obtained by sedimentation equilibrium of dTBG at pH 7.5 in 0.10 M phosphate and 0.10 M KCl, proving that removal of most of the sugar residues did not result in self-association.

The preparation of Dns-conjugated TBG has been described elsewhere (Johnson et al., 1980). The number of moles of bound Dns was 1.0 as determined by absorbance measurements at 340 nm (after correcting for the small amount of T_4 present in the TBG preparation) and using 3360 M^{-1} cm^{-1} as the molar extinction coefficient of the Dns group (Grimaldi et al., 1982).

Fluorescence spectra were obtained with a Perkin-Elmer spectrofluorometer (Model MPF-3). Temperature was controlled by circulating water from a controlled bath through a jacket surrounding the cuvette. Polarization measurements were performed in the same instrument. The polarization is defined as $(I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$ where I is the intensity of emission and $G = I_{hv}/I_{hh}$. The first and second subscripts refer to the plane of polarization of the excitation and emission beans, respectively (v = vertical, h = horizontal). The fluorescence intensity values for the two probes, ANS and Dns, were determined from $I = I_{vv} + 2I_{vh}$. The excitation and emission wavelengths for ANS were 380 and 470 nm, respectively, and for Dns were 340 and 490 nm, respectively. Tryptophan was excited at 280 nm, and its emission was measured at 340 nm.

The titration of the fluorescence of TBG and dTBG by T_4 was performed by adding 10-μL aliquots of T_4 in 0.10 M bicarbonate, pH 8.6, to a 1-mL solution of protein (40 μg/mL) containing 0.10 M Tris buffer, pH 8.0, in a 1 cm^2 cuvette.

Sialic Acid. Sialic acid released from TBG by enzymatic digestion was measured by the thiobarbituric acid method (Warren, 1959).

Enzymatic Digestion. To a solution of TBG (40 μg/mL) in 1 mL of 0.10 M phosphate, pH 6.0, was added 1 μL of mixed glycosidases (5 mg/mL) and α-mannosidase (1 unit/mL) in the same buffer. The reaction was followed in the fluorometer. A similar procedure was used for the neuraminidase reaction where 1 μL of the enzyme solution (100 units/mL) was added to 1 mL of the TBG solution (40 μg/mL).

Sedimentation Velocity. The determination of the sedimentation coefficient was performed at 20 °C by the method described by Attri & Minton (1984). We thank Dr. Attri for performing the measurements. The concentration of TBG was very low, i.e., 170 μg/mL, so that intermolecular (second virial) effects would be unimportant. We could therefore calculate the frictional coefficient (f) directly from the observed sedimentation coefficient and the known molecular weight (M) and partial specific volume ($\bar{v}$). The molecular frictional coefficient (f) is obtained from

$$f = \frac{M(1 - \bar{v}\rho)}{Ns}$$

The molar frictional coefficient of a sphere (f_0) of the same molecular weight is

$$f_0 = 6\pi\eta Nr = 6\pi\eta N\frac{(3M\bar{v})^{1/3}}{4\pi N}$$

where η is the viscosity and π the density of the solution.

The relaxation ratio is ρ_h/ρ_0 where ρ_h is the relaxation time and ρ_0 is the calculated relaxation time for a rigid, unhydrated sphere of the same mass, i.e., $\rho_0 = 3\eta\bar{v}M/RT$.

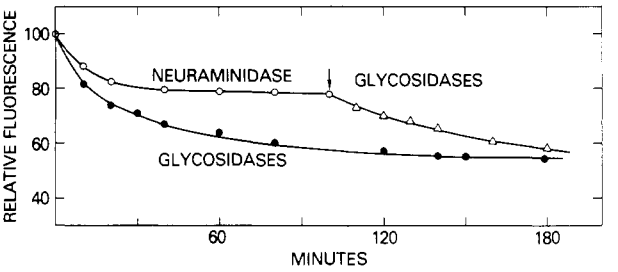

FIGURE 1: Rate of loss in Trp fluorescence intensity of TBG at 340 nm with glycosidic enzymes. The closed circles (●) show the effect of the mixed glycosidase preparation plus α-mannosidase. The open circles (○) show the effect of neuraminidase. The triangles (△) show the effect of adding the mixed glycosidases to TBG 100 min after the addition of neuraminidase. The reaction was performed at pH 6.0 in 0.10 M phosphate at 37 °C. The concentration of protein was 40 μg/mL.

RESULTS

Fluorescence Changes Accompanying Enzymatic Deglycosylation of TBG. (A) Intrinsic Emission. (1) Tryptophan. The rate of decrease in Trp emission intensity with enzymatic deglycosylation of TBG at pH 6.0 in 0.10 M phosphate is shown in Figure 1. The decrease observed in several preparations of TBG was close to 50% after several hours of hydrolysis. When neuraminidase was used instead of a mixture of glycosidases, the fluorescence decrease was about half as large (Figure 1). If the mixed glycosidase preparation was added 100 min after neuraminidase, the fluorescence decreased further to the level observed with only the glycosidases.

One mole of T_4 is bound to TBG with a constant of $\sim 10^{10}$ M^{-1} and quenches Trp emission by about 50% (Cheng et al., 1979; Robbins & Edelhoch, 1985). Interestingly, about the same extent of quenching occurs when 1 mol of T_4 is bound to dTBG (Figure 2). This result would be explained if some of the Trp residues that are quenched by removal of the carbohydrate groups are also quenched by T_4 in the native glycoprotein. It has been reported earlier that there is only about a 60% reduction in the binding constants of T_4 and anti-TBG antibody when TBG is deglycosylated (Cheng et al., 1979). The quenching curves of TBG and dTBG emission are linear with T_4 concentration and, therefore, in accord with their high affinity for T_4 (Figure 2). Most of the quenching of Trp emission by T_4 is probably the result of energy transfer from the Trp residues to the T_4 chromophore which absorbs strongly in the region of Trp emission (Perlman et al., 1968).

The loss in fluorescence of TBG with deglycosylation is not accompanied by a shift in its emission spectrum (Figure 3).

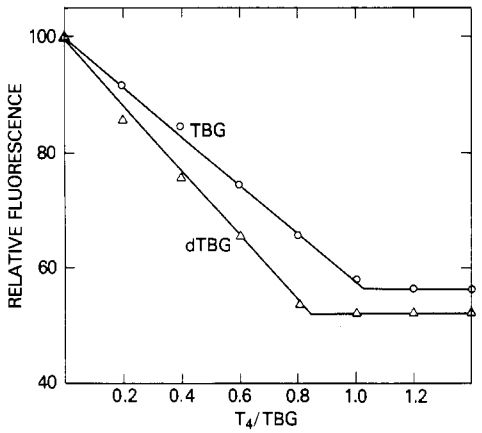

FIGURE 2: Titration and quenching of Trp fluorescence on addition of T_4 to TBG and dTBG. The abscissa shows the amount of T_4 added to the protein solution expressed as moles of T_4 per mole of protein. The titration was performed in 0.10 M Tris, pH 8.0 at 24 °C. The concentration of protein was 40 μg/mL.

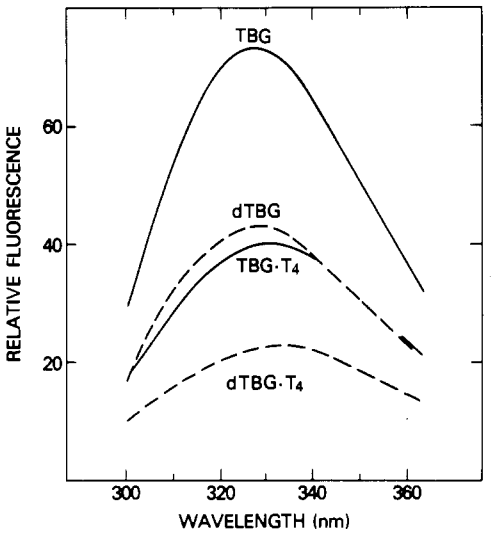

FIGURE 3: Emission spectra of TBG and dTBG before and after addition of 1 mol of T_4 per mole of protein. Conditions: pH 8.0, 0.10 M Tris, 24 °C.

There is a small red shift (329 → 333 nm) in the emission spectrum of native TBG upon addition of T_4 which is about twice as large with dTBG (Figure 3). These results suggest that the loss in fluorescence of TBG with deglycosylation originates equally from both surface and internal Trp residues. The larger red shift observed with T_4 binding to dTBG compared to TBG requires that a greater percentage of the fluorescence originating from internal residues of dTBG is quenched. One way to explain the larger shift in emission peak is by a change in the relative degree of quenching among the Trp residues by energy transfer to T_4. This could occur if a structural transition changed the steric relationships (distances and angles) between the four Trp residues and T_4. Further evidence for a molecular rearrangement of the Trp residues in TBG with deglycosylation comes from acrylamide quenching experiments (see below).

(*2*) *Effect of Deglycosylation on Trp Emission in the Presence of* T_4. Prior addition of 1 mol of T_4 to TBG prevents most or all of the loss in Trp fluorescence that is observed with deglycosylation (Table I). Only a few percent decrease was observed with T_4 present. However, the rate of deglycosylation, as measured by the liberation of sialic acid, was hardly affected (Table I). It has been reported that T_4 binding produces a conformational change in TBG (Grimaldi et al., 1982) which

Table I: Sialic Acid Content with Mixed Glycosidase Hydrolysis[a]

time	sialic acid liberated (nmol)		% decrease in fluorescence	
	TBG	T_4·TBG	TBG	T_4·TBG
10 min	5.1	4.0	25	4
25 min	7.0	6.5	31	4
24 h	8.2	8.0	39	4

[a] Protein concentration = 40 μg/mL.

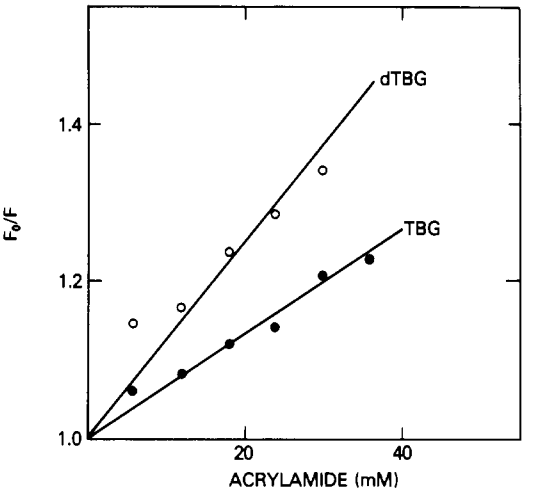

FIGURE 4: Quenching of fluorescence (F) of TBG and dTBG by acrylamide. Conditions: pH 8.0, 0.10 M Tris, 24 °C. F_0 is the fluorescence intensity in the absence of acrylamide. The concentration of protein was 40 μg/mL.

stabilizes the structure of TBG. This increase in stability is enough apparently to prevent the conformational change responsible for the quenching of Trp emission with deglycosylation. Presumably, the rate of the conformational change is now too slow to measure. In the absence of T_4, deglycosylation decreases the stability of TBG (see below).

(*3*) *Acrylamide Quenching of Trp Fluorescence.* In order to determine whether deglycosylation of TBG modifies the structure of TBG, we have measured the quenching effect of acrylamide on Trp emission (Figure 4). The Stern–Volmer equation for collisional quenching is

$$F_0/F = 1 + K_{sv}[\text{Acr}] = 1 + k_3\tau[\text{Acr}]$$

where K_{sv} is the Stern–Volmer constant, k_3 is the biomolecular collisional rate constant, and τ is the lifetime. The value of K_{sv} is about twice as large for dTBG as for TBG (Figure 4). Since the emission intensity of TBG declines about 50% with deglycosylation and represents a decrease in emission of both internal and external Trp residues, it is unlikely that the Trp residues that are quenched by acrylamide have a longer lifetime in dTBG. If the lifetimes of the Trp residues in dTBG stay the same or, more likely, decrease in accord with their reduced quantum yields, then the biomolecular collisional rate constant would increase significantly with deglycosylation. An increase in rate constant suggests that the Trp residues are more exposed to the solvent in dTBG than in TBG and implies some molecular rearrangement (or unfolding) with deglycosylation.

(*B*) *Extrinsic Emission.* (*1*) *Dns.* TBG was covalently labeled with Dns. Deglycosylation of Dns·TBG produced a 25% decrease in Dns polarization and a 30% decrease in Dns emission intensity (Figure 5). The large decrease in quantum yield of the Dns label suggests a change in its interaction with the protein with deglycosylation. The decrease in polarization indicates that there is a reduction in rotational relaxation time

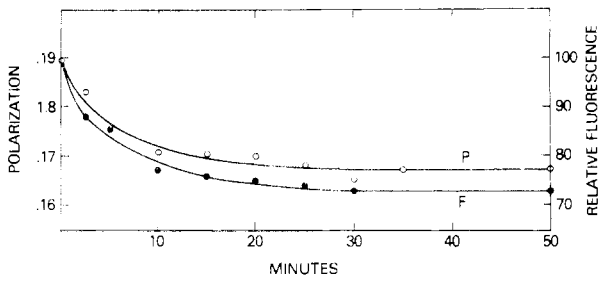

FIGURE 5: Rate of change in polarization (○) and fluorescence (●) of Dns in labeled TBG with digestion by the mixed glycosidase preparation plus α-mannosidase. Conditions: pH 6.0, 0.10 M phosphate, 37 °C. The protein concentration was 40 μg/mL. One mole of Dns was covalently conjugated to TBG. Wavelengths of excitation and emission were 340 and 490 nm, respectively.

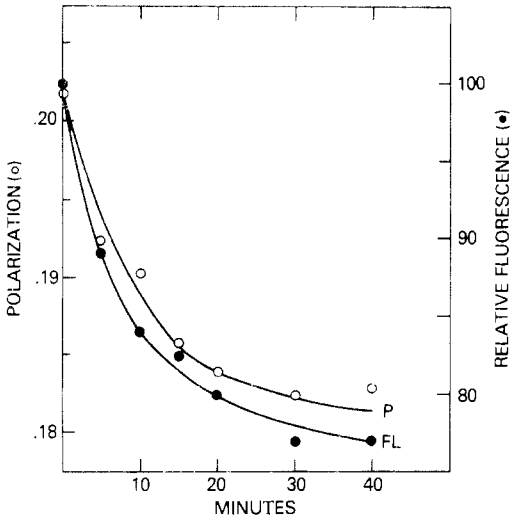

FIGURE 6: Rate of change in polarization (○) and fluorescence (●) of ANS with digestion by the mixed glycosidases plus α-mannosidase. Conditions: pH 6.0, 0.10 M phosphate, 37 °C. The protein concentration was 40 μg/mL. The solution contained 2 mol of ANS per mole of TBG. Wavelengths of excitation and emission were 380 and 470 nm, respectively.

with deglycosylation since it is unlikely that the lifetime increases when the fluorescence intensity of Dns decreases. If the carbohydrate chains are surrounded by solvent, they would impose a significant frictional drag on the protein, and their elimination could explain the smaller relaxation time for dTBG. If, however, they are residing on the surface of TBG in contact with amino acid residues, their release would reduce the polarization but would not explain the rather large decrease in polarization when coupled with a significant fall in lifetime. In fact, covalent dye labeling of the protein could offer a convenient method of following the rates of deglycosylating reactions of glycoproteins if the carbohydrate chains are appreciably solvated.

(*2*) *ANS.* ANS binds to TBG with a binding constant of $\sim 10^6$ with a large increase in its fluorescence intensity and a strong blue shift in its emission peak (Green et al., 1972). It is bound in the T_4 site since it is readily displaced, i.e., loses its fluorescence, by the addition of 1 mol of T_4. Deglycosylation of ANS·TBG resulted in a 20% decrease in ANS polarization and a 25% decrease in its fluorescence (Figure 6). Desialylation of ANS·TBG with neuraminidase resulted in a similar loss in ANS fluorescence as found with the glycosidase preparation (Figure 7). Evidently, the fluorescence of the ANS label reflects the change in structure corresponding to the loss of sialic acid residues and not to the remaining carbohydrates released by the mixed glycosidases.

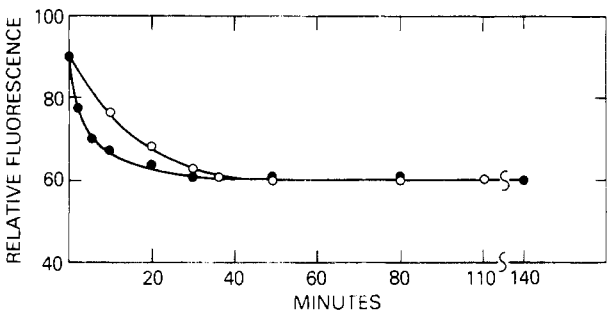

FIGURE 7: Rate of change in ANS fluorescence with digestion by neuraminidase (○) and by mixed glycosidases plus α-mannosidase (●). Conditions: pH 6.0, 0.10 M phosphate, 37 °C. Protein concentration was 40 μg/mL. The solutions contained 5 mol of ANS per mole of TBG. Wavelengths of excitation and emission were 380 of added ANS/TBG compared to 2 mol. A larger percentage of the sites are filled with 5 mol of ANS.

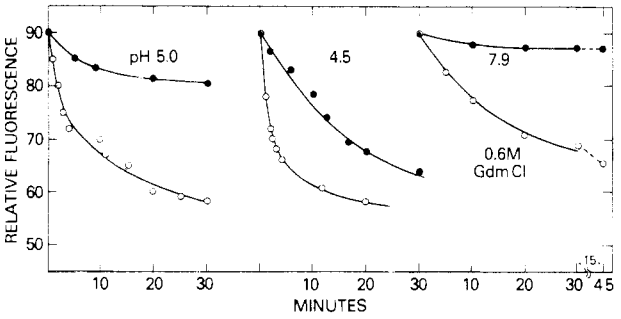

FIGURE 8: Comparison of the rate of loss in Trp fluorescence between TBG (●) and dTBG (○) at pH 5.0, 4.5, and 7.9. The pH 7.9 solutions contained 0.60 M GdmCl and 0.10 M Tris; 0.10 M acetate was the buffer used at pH 4.5 and 5.0. T = 24 °C.

The decrease in ANS polarization with deglycosylation indicates a reduction in relaxation time since the fluorescence intensity also decreases. However, as in the case of the Dns label, the loss of most of the carbohydrates probably only accounts for part of the reduction in the relaxation time of TBG.

Stability of dTBG. We have reported elsewhere that T_4 binding increases the stability of TBG to inactivation, i.e., loss of T_4 binding, by dilute acid and dilute GdmCl solutions (Grimaldi et al., 1982). Deglycosylation of TBG, however, reduces its stability to both sets of conditions. Thus, at pH 5.0, where TBG is still stable, dTBG undergoes a rapid loss in fluorescence (Figure 8). At pH 4.5, where TBG is slowly inactivated, dTBG loses Trp fluorescence at a much faster rate (Figure 8).

TBG is stable in dilute GdmCl solutions at pH 8.0 but is readily denatured at concentrations of about 1.5 M and higher (Grimaldi et al., 1982). In Figure 8, it can be seen that dTBG loses fluorescence rather rapidly in only 0.60 M GdmCl, whereas native TBG is stable. (Native TBG shows only a small decrease in Trp emission similar to that usually observed with the native protein in the absence of denaturant when followed in the fluorometer.)

The inactivation of TBG involves only a rather limited change in its structural parameters (Gershengorn et al., 1977b). The loss of most of the carbohydrates appears to modify the interactions among the amino acid residues to reduce the stability of the protein without significantly affecting the affinity for T_4.

Discussion

We have investigated the interaction of the four N-linked glycosidic chains of TBG with its apoprotein by evaluating the

changes in the fluorescence behavior of one intrinsic and two extrinsic probes. The quantum yields of all three types of chromophores decrease significantly when 86% of the carbohydrates are removed enzymatically. The reduced yields of all three probes, the tryptophans, the ANS in the T_4 site, and the DNS covalently distributed among the surface amino acid residues of TBG, cannot be accounted for by a trivial type of quenching or even by a small localized effect since the three types of probes are distributed in different parts of the protein. Furthermore, elimination of the sialic acid residues, which are located at the termini of the complex type of polysaccharides and represent only 10 of the 45 residues removed by the mixed glycosidases, resulted in a loss in Trp and ANS fluorescence. Since the sialic acid residues are separated from the N-linked asparagine group by five sugar residues, there should be no effect of its removal on the properties of the protein if they did not interact with the protein. It is likely that other sugar residues are also interacting with protein groups since desialylation has only about half the effect on the quantum yield of the Trp chromophores as that produced by removal of 86% of the carbohydrate residues. In contrast to the results with Trp fluorescence, both types of enzyme preparations had the same effect on the loss of ANS fluorescence. The localization of the ANS probe in the T_4 site evidently makes it less sensitive than the Trp probes which are more widely distributed in the structure of TBG.

The decrease in polarization of the ANS or Dns label with deglycosylation reflects a decrease in the relaxation time of dTBG. In part, this decrease represents the loss of 18% of the mass of the protein resulting from removal of 86% of the sugar residues. If the carbohydrates were maximally solvated (i.e., noninteracting), their removal would decrease the relaxation time much more than expected from the reduction in mass since they would exert a signficant viscous drag which would increase the frictional coefficient for both translational and rotatory motion. We have evaluated this effect on the translational motion by measuring the frictional coefficient of TBG and dTBG by sedimentation velocity experiments. Under identical conditions (pH 6.0, 0.10 M phosphate, and 20 °C), the sedimentation rates were 3.38 and 3.53 S, and the derived frictional ratios are 1.59 and 1.23 for TBG and dTBG, respectively, where M_r = 54 000 and 44 000 and $\bar{v}$ = 0.719 and 0.738, on the basis of amino acid and carbohydrate compositions (Cohn & Edsall, 1943; Gibbons, 1972).

The decrease in the frictional ratio of TBG with deglycosylation requires either an increase in molecular symmetry (i.e., shape or compactness) and/or a decrease in the degree of hydration. Both of these criteria would be simultaneously satisfied if the carbohydrate chains protruded, at least in part, into the bulk aqueous phase. If only the terminal sugar residues are in contact with the surface of the protein, we could explain both the polarization and frictional changes occurring with deglycosylation. Rupture of the interactions of sialic acid and other carbohydrate residues with amino acid residues would produce the minor transitions in the organization of the protein that we have observed by the changes in fluorescence parameters.

It is evident that two effects are influencing the fluorescent and hydrodynamic behavior of dTBG. The first is the loss of 18% of the mass of TBG. If these carbohydrate residues were not significantly solvated, the frictional (f/f_0) and relaxation (ρ/ρ_0) ratios of TBG would not change much, if at all. The decrease in f/f_0 and the apparent decrease in ρ/ρ_0 suggest that the carbohydrate chains are highly hydrated and produce a strong frictional drag on the motions of TBG. The second effect is the modification in the structure of the apoprotein as revealed by the changes in quantum yield. These, however, tell us almost nothing of the *nature* of the conformational changes. On the other hand, the acrylamide data indicate that the Trp residues become more exposed to the solvent with deglysylation and therefore are more hydrated. If the latter were the only effect, the sedimentation and polarization values would decrease. It is likely, therefore, that any unfolding due to the structural change in the protein is not large since, in that event, the sedimentation rate would have decreased. Further evidence against a major transition is that the binding constants for T_4 and antibody are only slightly affected (Cheng et al., 1979). Moreover, ANS, which is relatively weakly bound, continues to be bound after deglycosylation.

An interesting feature of the TBG molecule is that it undergoes irreversible denaturation and loss of T_4 binding in mild acid or dilute GdmCl (Gershengorn et al., 1977b; Johnson et al., 1980). The evidence presented here indicates that the oligosaccharide units in native TBG interact with the apoprotein moiety, affecting its structure and increasing its stability. This suggests that if the initial folding of the polypeptide chain occurred prior to its glycosylation then refolding of the mature, glycosylated molecule after inactivation may be prevented by the carbohydrate–protein interaction. Investigation of this possibility will be of interest.

Registry No. T_4, 51-48-9.

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Purification of the Calmodulin-Sensitive Adenylate Cyclase from Bovine Cerebral Cortex†

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ABSTRACT: A calmodulin-sensitive adenylate cyclase was purified 3000-fold from bovine cerebral cortex using DEAE-Sephacel, calmodulin–Sepharose, and two heptanediamine–Sepharose column steps. The purified enzyme activity was stimulated by calmodulin, forskolin, 5′-guanylyl imidodiphosphate, and NaF. The molecular weight of the protein component was estimated as 328 000 with a smaller form of M_r 153 000 obtained in the presence of Mn^{2+}. The most highly purified preparations contained major polypeptides of 150 000, 47 000, and 35 000 daltons on sodium dodecyl sulfate (SDS) gels. Photoaffinity labeling of the preparation with azido[^{125}I]iodocalmodulin gave one product of 170 000 daltons on SDS gels. It is proposed that the catalytic subunit of the calmodulin-sensitive enzyme is 150 000 ± 10 000 daltons and that the enzyme exists as a complex of one catalytic subunit and the stimulatory guanyl nucleotide regulatory complex. These data are consistent with the previous report that the catalytic subunit of this enzyme has a molecular weight of 150 000 ± 10 000 [Andreasen, T. J., Heideman, W., Rosenberg, G. B., & Storm, D. R. (1983) *Biochemistry 22*, 2757].

Adenylate cyclase sensitive to stimulation by calmodulin (CaM)[1] was first reported by Brostrom et al. in 1975, and independently in the same year by Cheung et al. (1975). In contrast to hormone-stimulated adenylate cyclase, which is present in almost every mammalian cell type (Ross & Gilman, 1980), CaM-sensitive adenylate cyclase has been demonstrated only in a limited number of tissues including brain, pancreatic islet cells, and adrenal medulla (Brostrom et al., 1975; LeDonne & Coffee, 1975; Valverde et al., 1979). Bovine cerebral cortex is believed to contain both CaM-sensitive and CaM-insensitive forms of adenylate cyclase (Brostrom et al., 1977; Westcott et al., 1979). It has been difficult to characterize the CaM-sensitive adenylate cyclases using membranes or unfractionated detergent-solubilized extracts because of the presence of calmodulin-insensitive forms of the enzyme and other CaM binding proteins. The ultimate characterization of CaM-sensitive adenylate cyclase and the elucidation of the regulatory mechanisms of the enzyme await the availability of a homogeneous preparation.

In this study, we describe a procedure for the partial purification of the CaM-sensitive adenylate cyclase which separates the enzyme from CaM-insensitive adenylate cyclase and several major CaM binding proteins. The subunit composition and molecular weight of the enzyme complex are reported.

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Materials and Methods

Materials

Bio-Gel A-5M was purchased from Bio-Rad. GppNHp was obtained from P-L Biochemicals. ATP, cAMP, and protein standards were from Sigma. [α-^{32}P]ATP and [^{3}H]cAMP were purchased from New England Nuclear and International Chemical Nuclear, respectively. All other reagents were of the finest available grade from commercial sources.

Methods

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the general method of Salomon et al. (1974) using [α-^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor product recovery. Assays contained in a final volume of 250 μL 20 mM Tris·HCl, pH 7.5, 1 mM [α-^{32}P]ATP (20 cpm/pmol), 5 mM theophylline, and 0.1% bovine serum albumin. All results are presented as the mean of triplicate assays with

[1] Abbreviations: CaM, calmodulin; N_s, stimulatory guanyl nucleotide regulatory complex of adenylate cyclase; N_i, inhibitory guanyl nucleotide regulatory complex of adenylate cyclase; GppNHp, 5′-guanylyl imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; IAP, islet activating protein from *Bordetella pertussis*; MABI, methyl azidobenzimidate; EDTA, (ethylenedinitrilo)tetraacetic acid; DMPC, dimyristoylphosphatidylcholine; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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