Effect of Deglycosylation on the Binding and Immunoreactivity of Human Thyroxine-binding Globulin

(Received for publication, March 2, 1979, and in revised form, May 22, 1979)

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Thyroxine-binding globulin (TBG), prepared from human serum by an improved purification method, was treated with a mixture of neuraminidase, β-galactosidase, o-mannosidase, and β-N-acetylglucosaminidase, which resulted in the removal of approximately 86% of saccharides. Purification by thyroxine-Sepharose affinity chromatography gave a homogenous protein as shown by equilibrium sedimentation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid and N~2-teram sequence analysis indicated that the protein moiety was intact. Deglycosylation had no effect on the stoichiometry of the binding of L-thyroxine as shown by tryptophanyl fluorescence quenching and equilibrium dialysis at pH 8.6 and 25°C. However, the affinity constant for L-thyroxine was reduced from 1.6 × 10^9 M^-1 to 0.58 × 10^9 M^-1. Analysis of radioimmunoassay data revealed that deglycosylation resulted in a slight decrease of the affinity constant for anti-TBG antibody from 3.9 × 10^10 M^-1 to 1.8 × 10^10 M^-1. These results suggest that the polypeptide moiety, rather than the heterosaccharides, contains the antigenic determinants. Removal of the majority of the heterosaccharides of TBG has only a minor effect on its immunoreactivity and on the binding of thyroid hormone.

Although all studied serum proteins except albumin and thyroxine-binding prealbumin are glycoproteins containing covalently bound heterosaccharide groups, very little is known about the structure and function of their carbohydrate moieties (1, 2). Among the three proteins which transport the thyroid hormones in human serum, thyroxine-binding globulin (TBG) has the highest affinity for L-thyroxine (T₄) and carries more than 76% of thyroid hormone in the serum. It is a glycoprotein with a reported carbohydrate content of 7.5 to 32% (3-6). Recently, Zinn et al. (5, 6) partially isolated and characterized the glycopeptides from TBG and reported a carbohydrate content of 14.6% based on a molecular weight of 63,000. Although the sialic acid in TBG has been shown to be involved in retarding its clearance from plasma (6-8), no data are available regarding the role of the heterosaccharides in the ability to elicit antibody production or in the binding of thyroid hormone. Recently, there has been an increasing interest in studies on the biosynthetic pathway and the identification of the precursors of the biologically important glycoproteins. Anti-glycoprotein antibodies are frequently used in the isolation and identification of the in vitro biosynthetic products (9).

The information derived from the study of the effect of deglycosylation on the immunoreactivity of glycoproteins will be valuable for the analysis of the biosynthetic entities. Several reports have described the effect of the removal of carbohydrates on the activity of several enzymes (10-12), and on the antiviral activity of human interferon (13). Our study is the first report on the effect of deglycosylation on the binding ability of a transport protein.

EXPERIMENTAL PROCEDURES

Materials—Fresh human plasma was obtained from the blood bank of the National Institute of Health. L-[¹²⁵I]T₄ (950 pCi/µg) was purchased from New England Nuclear and nonradioactive L-T₄ from Calbiochem. Sepharose 4B and DEAE-Sephadex A-50 were from Pharmacia. The mixture of glycosidases prepared (14) from a strain of Diplococcus pneumoniae, type 1, was kindly provided by G. Ashwell, National Institute of Arthritis, Metabolism, and Digestive Diseases. α-Mannosidase (1 unit/ml) was purchased from Miles Biochemicals. Sodium dodecyl sulfate was obtained from BDH Chemicals, Ltd.

Preparation of TBG—TBG was purified by an improved two-step procedure derived from the three-step purification described by Gershenorn et al. (15). The use of α-Sepharose affinity chromatography as the first step was the same as before. In the present method, a shallower gradient was used in the subsequent DEAE-Sephadex A-50 chromatography. A linear gradient of 0.077 M to 0.12 M KCl in 0.06 M Tris with a conductance of 6.3 and 9.4 mmho, respectively, resulted in a homogeneous preparation of TBG as determined by polyacrylamide gel electrophoresis. The elimination of Sephadex G-150 gel filtration as the third step in the purification gave an overall yield of 50 to 65% as compared to 25% as originally reported (15) and reported in other laboratories (4, 5, 16, 17).

Preparation of Deglycosylated Thyroxine-binding Globulin (D-TBG) A preliminary attempt to deglycosylate TBG using endo-α-β-N-acetylglucosaminidase H purified from Streptomyces gisaeus was unsuccessful. This is explained by the recently reported structure of the TBG glycopeptidol (8) since this enzyme only attacks neutral oligosaccharide chains with high mannose content (11). Therefore, it was necessary to use mixed glycosidases. A solution of TBG (8.2 mg) in 4.2 ml of 0.1 M sodium phosphate (pH 6.2) was incubated with 80 µl of mixed glycosidases (10 mg/ml) and 40 µl of α-mannosidase (1 unit/ml) at 37°C for 24 h. The mixture was then applied to a Tr-Sepharose column (1.2 × 8 cm). After the column was washed with 60 ml of 0.1 M KHCO₃ (pH 7.8), the D-TBG was eluted with 10 mM KOH. The 1 ml eluant was collected into 1 ml of 0.5 M sodium phosphate (pH 7.8). A control experiment indicated that the incubation of TBG under these conditions in the absence of glycosidases had no effect on the T₄ binding of native TBG as measured by tryptophanyl fluorescence titration or equilibrium dialysis (see below). TBG and α-TBG concentrations were routinely determined by their absorption at 280 nm. E₁₅₀ = 6.2 (15) and 7.45 were used for...
TBG and d-TBG, respectively. The latter value was calculated (18) from the amino acid composition (see below) using 9 and 4 residues for tyrosine and tryptophan, respectively. The absorption at 280 nm was corrected for the small amount of Td present, as previously described (15).

Analytical Ultracentrifugation—Ultracentrifugation was performed in double-sector cells at 24°C in a Spinco model E ultracentrifuge equipped with ultraviolet scanner optics and temperature control by an RTIC unit. Solutions of d-TBG (0.32 to 0.726 mg/ml) were prepared in 0.1 M NaCl, 0.1 M sodium phosphate, pH 7.5. Equilibrium was achieved after 48 h at 20,000 rpm. Weight average molecular weights were calculated from the slopes of ln C against R^2. The partial specific volume (0.738) was calculated from the amino acid and carbohydrate composition (see below) and their known partial specific volume (19, 20).

Carbohydrate Analysis—Hexosamine was determined with a Duram amino acid analyzer. Samples of d-TBG were hydrolyzed in 3 M HCl at 105°C for 2 h and correction was made for loss during hydrolysis.

Sialic acid was determined by the thiobarbituric acid method (21). Protein samples were hydrolyzed in 0.1 N H2SO4 at 80°C for 1 h. Neutral sugars were identified and quantitated by partition chromatography as described (15).

Amino Acid and NH2-terminal Analysis—Amino acid analyses were performed according to the method of Spackman et al. (22). The samples were hydrolyzed with constant boiling HCl in evacuated, sealed tubes at 105°C for 24 h.

NH2-Terminal sequence analysis was performed as described (23). The phenylthiohydantoins were identified and quantitated by gas-liquid partition chromatography and mass spectrometry.

Equilibrium Dialysis—Equilibrium dialysis was carried out in 1 ml acrylic plastic cells. The cellulose dialysis membrane was boiled for 30 min in 0.2 M NaCO3, 1 mm EDTA, and then washed extensively with distilled water. Protein concentrations ranged from 0.5 to 1 × 10^-6 M. The molecular weights of TBG and d-TBG were taken as 54,000 (15) and 44,000, respectively. Dialysis cells were shaken gently for 24 h at 25°C. The binding constants were determined according to the following equation:

\[ Y_T = \frac{n_T r_CT}{1 + K_T r_CT} \]

where K intake is the apparent association constant for the binding of T to TBG, r CT is the molar concentration of free T4 in the solution, and r CT is the apparent number of binding sites, and r CT is the molar ratio of bound T4 to total protein. The "best fits" were accomplished with an on-line modeling program, MLAB, developed at the National Institutes of Health (24). This program utilizes the Marquardt-Levenberg algorithm to perform a nonlinear least-squares fit of data to a specific function (25). Reported errors of fitted parameters correspond to approximately 1 S.D.

Radioimmunoassay—The anti-TBG antiserum was obtained as described (26). Radioiodination of TBG was performed by the chloramine-T method (27, 28) and the iodinated TBG was purified as described by Gershengorn et al. (26). Radioimmunoassay was performed as described by Levy et al. (29) with minor modifications. The anti-TBG serum was used at a 1:20,000 final dilution. The first incubation with anti-TBG was carried out for 48 h at 4°C, and the second incubation with anti-rabbit IgG for 24 h at 4°C. Unlabeled purified TBG and d-TBG were added at serial dilution from 1 to 50 ng/ml.

The affinity constant and binding capacity of antiserum for TBG and d-TBG were estimated by a computer program, "SCAFIT," as described by Fader and Rodbard (30), and also a newly developed computer program by Munson and Rodbard2 using a model for two ligands competing simultaneously for one type of binding site.

Electrophoresis—Polyacrylamide gel electrophoresis was performed as before (15). Molecular weight determination using sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (31).

Fluorescence Titration—Fluorimetric titration of TBG with T4 was performed at 25°C in a Perkin-Elmer spectrofluorometer. The excitation and emission wavelengths were 280 nm and 340 nm, respectively. The buffer used was 0.06 M Tris, 0.1 M KCl, pH 8.6. The initial protein concentration was 0.79 μM. Increments of 10 μl of T4, with a concentration of 18.7 μM were added. The readings were corrected for dilution.

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2 P. J. Munson and D. Rodbard, manuscript in preparation.

FIG. 1. Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis of native (left) and deglycosylated TBG (right). Gels were stained with Coomassie blue.
S.D. for three experiments). The partial specific volume was calculated from the amino acid and carbohydrate composition (Tables I and II). As shown in Fig. 2, no significant deviation from linearity could be detected over the entire range of the In C versus R² plot.

A molecular weight of 43,700 was obtained from dodecyl sulfate-polyacrylamide gel electrophoresis. According to Frank and Rodbard (32), a criterion for the validity of a molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis is the presence of a common point of intersection of the Ferguson plots when total gel concentration (% T) is near zero. Fig. 3 shows that the plots for three reference proteins and for D-TBG intersect at a common point of 0% T. The molecular weight of D-TBG calculated on the basis of log molecular weight versus RF is in excellent agreement with that obtained from sedimentation equilibrium studies.

TBG was found to contain 21% carbohydrate. Removal of 86% of sugar residues as determined by sugar analysis gives a calculated molecular weight of 44,000, based on the known molecular weight of TBG (15) and subtraction of the mass of sugars. The value is virtually identical with those determined by sedimentation equilibrium studies and dodecyl sulfate-polyacrylamide gel electrophoresis.

**Binding of T₄ to TBG and D-TBG**

The effect of deglycosylation of TBG on the binding of T₄ was assessed by tryptophanyl fluorescence quenching and by equilibrium dialysis.

**Tryptophanyl Fluorescence Quenching**—Fig. 4 shows the quenching of tryptophanyl fluorescence by the titration of T₄ to TBG and D-TBG. Both the native and deglycosylated proteins in the present study contained 0.12 mol of T₄ as determined by absorption at 325 nm (15). In accord with earlier reports (33, 34), the maximal quenching (60% of the tryptophanyl fluorescence intensity) occurred at a molar ratio of T₄ to TBG of 1.0 (0.88 mol of added T₄ plus 0.12 mol present in the preparation). The same result was obtained for D-TBG. Thus, the stoichiometry of the binding of T₄ to both proteins is 1:1.

**Equilibrium Dialysis**—The data for the binding of [¹²⁵I]T₄ to D-TBG and TBG at pH 8.6 are illustrated in Fig. 5. The lines are the theoretical curves calculated from Equation 1. The data fit the calculated curves to give n = 1.01, K = 5.8

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### Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>TBG residues/100,000 g amino acids</th>
<th>D-TBG residues/100,000 g amino acids</th>
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</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>66</td>
<td>66</td>
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<td>Histidine</td>
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<td>27</td>
</tr>
<tr>
<td>Arginine</td>
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<td>14</td>
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<tr>
<td>Aspartic acid</td>
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<td>87</td>
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<tr>
<td>Threonine</td>
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<td>60</td>
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<tr>
<td>Serine</td>
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<td>70</td>
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<tr>
<td>Glutamic acid</td>
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<td>102</td>
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<td>Proline</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Alanine</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Alanine</td>
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<td>N/Cystine</td>
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<td>12</td>
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<td>Isoleucine</td>
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<tr>
<td>Phenylalanine</td>
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<tr>
<td>Tryptophan</td>
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<td>10</td>
</tr>
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</table>

*Values were obtained from earlier work (15).*

### Table II

<table>
<thead>
<tr>
<th>Sugar</th>
<th>TBG residues/mol</th>
<th>D-TBG residues/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Mannose</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Total sugar (%)</td>
<td>21</td>
<td>3</td>
</tr>
</tbody>
</table>

*Based on the molecular weight of 54,000.
* Based on the molecular weight of 44,000.
* Values were from previous report (15).
The procedure for the purification of human TBG has been reported from several laboratories (4, 5, 15, 17). Most of these utilized T,-Sepharose affinity chromatography as the first step followed by two or more chromatographic manipulations or preparative gel electrophoresis. This not only resulted in low yield (from 9.9% as reported by Nilsson and Peterson (4), to 20 to 35% reported by others (5, 15, 17)) but sometimes gave a protein preparation which had partially lost its binding capacity (37). Since it is known that TBG is readily denatured and loses its binding ability easily (34), it is desirable to seek a shorter procedure for purification. By lowering the initial KCl concentration from 0.09 M previously reported (15) to 0.077 M in the elution gradient of the DEAE-Sepharose A-50 chromatography, a homogeneous preparation was obtained. The overall yield from the present two-step purification was increased from approximately 25% reported earlier (15) to 50 to 55%.

The binding constants of the antibody for TBG and D-TBG were estimated to be $5.72 \times 10^{-10} \text{ M}^{-1}$ and $1.87 \times 10^{-9} \text{ M}^{-1}$, respectively. These values are consistent with those obtained by "SCAFIT" analysis. Therefore, it is clear that removal of the sugar moieties reduced the affinity of TBG to the immunoglobulins to about one-third of the native value.

**DISCUSSION**

The carbohydrate composition reported in Table II differed from the values found previously (15). Mannose had a higher value and glucose was not found in the present preparations purified by the two-step procedure. Earlier, Korcek and Ta-
tertiary structure or both is currently under study. However, Zinn et al. (5) recently reported that a variable amount of glucose was detected in the glycoprotein preparations but never in the isolated glycopeptides and suggested that glucose may be a contaminant. It is likely that the glucose detected previously was derived from Sephadex G-150. In the present procedure, Sephadex G-150 chromatography was eliminated and furthermore, the DEAE-Sephadex A-50 column was extensively washed before the elution gradient started.

Tarentino et al. (11) reported that the solubility of bovine pancreatic ribonuclease B and bovine deoxyribonuclease A was altered when their heterosaccharide chains were removed by endo-β-N-acetylglucosaminidase. A change in the solubility was also found by Leavitt (38) for the nonglycosylated glycoproteins of vesicular stomatitis virus and Sindbis virus. We also observed that p-TBG appeared to have a lower solubility at neutral pH in aqueous solution than that of TBG. Consequently, the binding studies were carried out in a slightly alkaline medium.

The affinity constant for T binding to TBG has been determined at physiological pH by many investigators with values ranging from 2.25 X 10^8 M^-1 to 9.5 X 10^7 M^-1 (4, 39-41). Our finding of an affinity constant of 1.6 X 10^7 M^-1 at pH 8.6 is lower than those at pH 7.4. This result is consistent with the report by Korcek and Tabachnick (34) in which binding at pH 8.6 was reduced to 25% of the value at pH 7.4.

Analysis of the binding data of D-TBG to thyroid hormone and anti-TBG antibody showed that the binding activity and immunoreactivity were not severely affected by 86% deglycosylation. It was found from both tryptophanyl fluorescence quenching and equilibrium dialysis that the stoichiometry of binding of T, had not been altered. However, the affinity was slightly reduced from 1.6 X 10^7 M^-1 to 0.58 X 10^6 M^-1. Furthermore, removal of the majority of the heterosaccharides only shifted the antigen-antibody displacement curve slightly, owing to a 2-fold lower affinity constant. This strongly suggests that the protein moiety, rather than the heterosaccharides, contributes to the antigenicity of the TBG. This result is similar to that obtained from the study of chicken ovalbumin, in which no evidence was found that the carbohydrates were the antigenic determinant (42). Our study further indicates that anti-D-TBG antibody can be used in the identification and purification of biosynthetic precursors of TBG devoid of, or with incomplete, sugar attachment.

The difference in the two slopes in the logit-log plot for the interaction of TBG or n-TBG with antibody is small, but is statistically significant. This suggests that there is a minor difference in the antigenic determinants of these two proteins. It is known that both the amino acid sequence and the conformation of a polypeptide contributes to the antigenicity. In view of the absence of proteolytic activity in the glycosidases used, it seems more plausible to explain the difference by a minor conformational alteration of the surface of TBG through the removal of the heterosaccharide. The reduction of the affinity of interaction of D-TBG with anti-TBG antibody probably also reflects such a change. Wang and Hirs (10) have shown, by spectrophotometric titration, that the heterosaccharide side chains of porcine pancreatic ribonuclease exert a stabilizing influence on the structure around at least two tyrosines located at or near the surface of the molecule. Since it was proposed by Zinn et al. (6) that there are four heterosaccharide chains of varying length attached to TBG, it is reasonable to assume that removal of these chains may have a similar destabilizing effect on D-TBG. Whether these changes are due to a partial alteration in the secondary or tertiary structure or both is currently under study.

Acknowledgments—We wish to thank Dr. T. Fairwell for valuable help with the automatic sequence analysis, and Dr. G. Ashwell for supplying us with the mixed glycosidases and for stimulating discussions. We are also grateful to Drs. D. Rodbard and P. J. Munson for providing assistance with the computer analysis for the determination of affinity constants of D-TBG to anti-TBG antibody.

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