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)

Sheue-yann Cheng,† Stefania Morrone,§ and Jacob Robbins
From the Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

Thyroxine-binding globulin (TBG), prepared from human serum by an improved purification method, was treated with a mixture of neuraminidase, β-galactosidase, α-mannosidase, and β-N-acetylglucosaminidase, which resulted in the removal of approximately 86% of saccharides. Purification by thyroxine-Sepharose affinity chromatography gave a homogeneous protein as shown by equilibrium sedimentation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid and NH2-terminal 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 $1.6 \times 10^9$ M$^{-1}$ to $0.58 \times 10^9$ M$^{-1}$. Analysis of radioimmunoassay data revealed that deglycosylation resulted in a slight decrease of the affinity constant for anti-TBG antibody from $3.9 \times 10^9$ M$^{-1}$ to $1.8 \times 10^9$ 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 75% 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 Institutes of Health. L-[$^{131}$I]T4 (950 μCi/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 Gershengorn et al. (15). The use of T4-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 KC1 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 ~95% as originally reported (15) and reported in other laboratories (4, 5, 16, 17).

Preparation of Deglycosylated Thyroxine-binding Globulin (DG-TBG) A preliminary attempt to deglycosylate TBG using endo-β-N-acetylglucosaminidase H purified from Streptomyces gрисев was unsuccessful. This is explained by the recently reported structure of the TBG glycopeptide (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 (82 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 T4-Sepharose column (1.2 x 8 cm). After the column was washed with 60 ml of 0.1 M Tris with a conductance of 6.3 and 9.4 mmho, the mixture was eluted with 10 ml 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 binding of native TBG as measured by tryptophanyl fluorescence titration or equilibrium dialysis (see below).

TBG and n-TBG concentrations were routinely determined by their absorption at 280 nm. $E_{1%}^{1%} = 6.2$ (15) and 7.45 were used for
Deglycosylated Thyroxine-binding Globulin

RESULTS

Homogeneity and Purity of d-TBG

The homogeneity of the product was analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis and by sedimentation equilibrium studies. Dodecyl sulfate-polyacrylamide gel electrophoresis showed that d-TBG consists of a single component (Fig. 1). In sedimentation equilibrium, a linear dependence of ln C versus R² was found as shown in Fig. 2. The purity of d-TBG was further demonstrated by NH₂-terminal sequence analysis. Six steps of Edman degradation were carried out by an automated sequencer. The NH₂-terminal alanine was determined as the phenylthiohydantoin by gas chromatography. Based on the molecular weight of 44,000 (see below, molecular weight determination), the initial yield was 60% and the extrapolated yield was 96%. Moreover, no other identifiable phenylthiohydantoin was present in the first degradation step. The fact that the same sequence, Ala-Ser-Pro-Glu-Gly-Lys, was found for d-TBG as for TBG (15) indicated that there was no proteolytic activity present in the exoglycosidases used. Amino acid analysis (Table I) gave the same ratio as that of TBG (15), which further substantiated that the enzymatic treatment only resulted in removal of the carbohydrate and that the protein portion of the molecule remained intact.

Carbohydrate Analysis

The carbohydrate content of d-TBG is listed in Table II. The sugar residues from TBG are included for comparison. Only 5 and 2 residues of glucosamine and mannose, respectively, per mol of d-TBG were found. This indicated that all sialic acid and galactose were successfully removed by enzymatic treatment. The content of carbohydrate in d-TBG, based on the molecular weight of 44,000, is 3%. As compared with a sugar content of 21% in TBG, 86% of carbohydrate had been removed from the parent protein.

The values for neutral sugars were obtained under the conditions which gave the maximal release from the TBG. Hydrolysis loss was corrected as described before (15).

Molecular Weight of d-TBG

The molecular weight obtained from two different preparations of d-TBG by sedimentation equilibrium studies using a partial specific volume of 0.738 was 43,700 ± 1,800 (mean ±

**Table II.** The carbohydrate content of d-TBG is listed in Table II. The sugar residues from TBG are included for comparison. Only 5 and 2 residues of glucosamine and mannose, respectively, per mol of d-TBG were found. This indicated that all sialic acid and galactose were successfully removed by enzymatic treatment. The content of carbohydrate in d-TBG, based on the molecular weight of 44,000, is 3%. As compared with a sugar content of 21% in TBG, 86% of carbohydrate had been removed from the parent protein.

The values for neutral sugars were obtained under the conditions which gave the maximal release from the TBG. Hydrolysis loss was corrected as described before (15).

Molecular Weight of d-TBG

The molecular weight obtained from two different preparations of d-TBG by sedimentation equilibrium studies using a partial specific volume of 0.738 was 43,700 ± 1,800 (mean ±

**Figure 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 1 TBG (1b) 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 TABLE I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>TBG[^a]</th>
<th>D-TBG[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>Histidine</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>Arginine</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>87</td>
<td>87</td>
</tr>
<tr>
<td>Threonine</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Serine</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>100</td>
<td>102</td>
</tr>
<tr>
<td>Proline</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Glycine</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>Alanine</td>
<td>96</td>
<td>94</td>
</tr>
<tr>
<td>% Cystine</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Valine</td>
<td>65</td>
<td>63</td>
</tr>
<tr>
<td>Methionine</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Leucine</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>53</td>
<td>49</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

[^a]: Values were obtained from earlier work (15).

TABLE II

<table>
<thead>
<tr>
<th>Sugar</th>
<th>TBG[^b]</th>
<th>D-TBG[^b]</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>

[^b]: Based on the molecular weight of 54,000.
[^c]: Based on the molecular weight of 44,000.
[^d]: Values were from previous report (15).

TBG or D-TBG with T₄. 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.
data which were linear over the entire range used. The slopes obtained for TBG and D-TBG were calculated to be $3.94 \times 10^{-9} \text{ M}^{-1}$ and $8.17 \times 10^{-11} \text{ M}^{-1}$ for D-TBG and TBG, respectively. D-TBG and TBG have the same number of binding sites for $T_4$. However, removal of the carbohydrate lowered the binding affinity for $T_4$ to about 30% of the native value.

**Immunoreactivity of D-TBG**

The effect of deglycosylation on the immunoreactivity of TBG was examined by radioimmunoassay using rabbit antiantigen. Fig. 6 shows the logit-log plot of the data which were linear over the entire range used in the experiment. The data were also analyzed by weighted least squares linear regression with Student's $t$ test for identity of slopes. The slopes obtained for TBG and D-TBG were $-1.17 \pm 0.025$ (mean $\pm$ S.D.) and $-1.27 \pm 0.022$, respectively. Although the slopes of the lines were similar, they appeared to differ significantly ($p < 0.05$) in view of their small standard errors.

The affinity constant and binding capacity of the antiserum for native TBG were estimated first using a computer program `SCAFIT` (30) which provides an unweighted nonlinear regression analysis of bound/free versus total ligand. The affinity constant and the binding capacity of antibody for TBG were calculated to be $3.94 \times 10^{-9} \text{ M}^{-1}$ and $8.17 \times 10^{-11} \text{ M}^{-1}$, respectively. This program also provides an estimate of $C_{50}$ for the ligand. The values of $7.16 \pm 0.12 \text{ ng/ml}$ and $12.68 \pm 0.22 \text{ ng/ml}$ (mean $\pm$ S.D.) were obtained for TBG and D-TBG, respectively. With these two $C_{50}$ values, the ratio of the affinity constants of TBG and D-TBG can be estimated (35) from

$$K_1 = \frac{R}{h_1 (1 + R) - R}$$

Where $K_1$ and $K_2$ are the association constants of anti-TBG antibody for TBG and D-TBG, respectively; $h_1$ and $h_2$ are the $C_{50}$ values for TBG and D-TBG, respectively. $R$ is the bound/free ratio at $C_{50}$ for TBG. From these values, one can estimate the ratio of the $K$ values to be 2.24, thus the affinity constant of D-TBG for anti-TBG antibody was estimated as $1.76 \times 10^{-10} \text{ M}^{-1}$.

A more rigorous approach is to fit the displacement curves for TBG and D-TBG simultaneously using a model for a two-ligand, one-type of binding site. We employed a newly developed computer program (33) for this purpose, based on the principles developed by Feldman (36). This program provides a better utilization of all available data, rather than being limited to a narrow central region of the curves. By this approach, the binding constants of the antibody for TBG and D-TBG were estimated to be $5.72 \times 10^{-10} \text{ M}^{-1}$ and $1.87 \times 10^{-10} \text{ M}^{-1}$, 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 procedure for the purification of human TBG has been reported from several laboratories (4, 5, 15, 17). Most of these utilized $T_4$-Sepharose affinity chromatography as the first step followed by two or more chromatographic manipulations or preparative gel electrophoresis. This not only resulted in lower 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 (33) 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-Sephadex 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%.

D-TBG was prepared by reaction of purified TBG with mixed glycosidases followed by affinity chromatography. The homogeneity of D-TBG was demonstrated by dodecyl sulfate-polyacrylamide gel electrophoresis and sedimentation equilibrium studies. In order to ensure that the protein moiety was intact, NH$_2$-terminal sequence and amino acid analysis were carried out. The ratios among the amino acid residues were the same as obtained with TBG. Quantitative NH$_2$-terminal analysis by Edman degradation yielded 0.98 mol of alanine/mol of protein. Furthermore, six steps of the Edman degradation at the NH$_2$-terminal region of D-TBG gave the same sequence as that obtained with TBG. Therefore, the treatment with exoglycosidases was not accompanied by proteolysis and was limited to the removal of the heterosaccharide chains.

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-
bachnik (17) found 2 residues of glucose/mol of their TBG preparations. 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.

Tarenino 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-ß-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 d-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 12 binding to TBG has been determined at physiological pH by many investigators with values ranging from 2.24 X 108 M-1 to 9.5 X 107 M-1 (4, 39-41). Our finding of an affinity constant of 1.6 X 107 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 tryptophan fluorescence quenching and equilibrium dialysis that the stoichiometry of binding of T4, had not been altered. However, the affinity was slightly reduced from 1.6 X 107 M-1 to 0.58 X 107 M-1. Furthermore, removal of the major part 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 d-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 contribute 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.

REFERENCES

Deglycosylated Thyroxine-binding Globulin

Effect of deglycosylation on the binding and immunoreactivity of human thyroxine-binding globulin.
S Cheng, S Morrone and J Robbins


Access the most updated version of this article at http://www.jbc.org/content/254/18/8830

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/18/8830.full.html#ref-list-1