Binding of Thyroglobulin to Bovine Thyroid Membranes

ROLE OF SPECIFIC AMINO ACIDS IN RECEPTOR RECOGNITION*

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Bovine thyroglobulin was treated with increasing ratios of succinic anhydride, trinitrobenzene sulfonic acid, tetranitromethane, and N-acetylimidazole in an attempt to assess the role of lysine or tyrosine residues in binding to thyroid membrane receptors. Extensive succinylation results in dissociation to $12\,S$ thyroglobulin with retention of a considerable portion of the three-dimensional structure. Only 25% of the lysine residues can be modified by trinitrophenylation without affecting inter-subunit interactions. Succinylation as well as trinitrophenylation increases the affinity of thyroglobulin for the membrane receptor by a factor of 2. The binding of thyroglobulin to the membrane was reduced after nitration of 30% of the tyrosyl residues with tetranitromethane. O-Acetylation of 40–70% of the tyrosyl residues by N-acetylimidazole nearly abolished the ability of thyroglobulin to bind to the membrane. Removal of the O-acetyl group with hydroxylamine restored the binding properties. The results indicate that tyrosyl residues play an important role in thyroglobulin interactions with thyroid membranes.

Thyroglobulin, which represents 75% of the total sedimenting components of the thyroid gland (1), is a high molecular weight glycoprotein ($M_\text{r} = 660,000$) which is the precursor of the thyroid hormones triiodothyronine and thyroxine. The physicochemical properties of thyroglobulin have been studied extensively (2, 3) and the sequence of sugars in the carbohydrate moieties has been elucidated (4, 5). More recent studies have been directed toward an understanding of the synthesis, storage, and degradation of thyroglobulin (3, 6–12).

The specific interaction of thyroglobulin with specific receptors on thyroid membranes may be a controlling factor in regulating the biosynthesis and biodegradation of the iodoprotein. Using techniques employed in the study of hormone-receptor interactions, Consiglio et al. (13) reported that bovine thyroglobulin will bind to a specific receptor on thyroid plasma membranes.

The present report is part of a study in which we are attempting to elucidate the structural features of thyroglobulin which are involved in binding to the thyroid receptor. The importance of the carbohydrate chain in binding is the subject of the accompanying report (14). The results to be presented here are part of a study concerned with a determination of the role of amino acid side chains (lysine and tyrosine) in binding to the receptor.

Two derivatives of each of the two amino acids were prepared and their physicochemical properties were studied prior to their being radioiodinated for the binding studies. Modification of the lysyl residues by reaction with either succinic anhydride or trinitrobenzene sulfonic acid increases the affinity for the receptor. However, modification of the tyrosine residues either by nitration with tetranitromethane or by acetylation with N-acetylimidazole drastically reduces the affinity for the receptor.

These results, which implicate a role for tyrosine residues in the receptor recognition process, are consistent with the results in the accompanying report (14), in which a correlation was demonstrated between the recognition of N-acetylgalcoamine residues of the carbohydrate chain and the degree of iodination of thyroglobulin. The results in the present report show that the site of thyroglobulin iodination, namely, tyrosine residues, are, in fact, important determinants in the receptor-binding process.

MATERIALS AND METHODS

Bovine thyroglobulin was isolated by ammonium sulfate precipitation followed by gel filtration over Bio-Gel A-5m (15).

Succinyl-thyroglobulin was prepared by dissolving 35 mg of thyroglobulin in 2 ml of 0.1 M sodium bicarbonate buffer, pH 8.0. Succinic anhydride was dissolved in acetonitrile (0.1 g/ml). Ten microliters of the succinic anhydride solution was added to 2 ml of thyroglobulin and the pH maintained at 8.0 by the addition of sodium hydroxide. This quantity of succinic anhydride corresponded to a ratio of succinic anhydride to lysine of 0.5:1. Five hundred microliters of the reaction mixture was withdrawn and put aside at room temperature and marked "1:2." A sufficient amount of succinic anhydride was added to the remaining 1.5 ml of the mixture to bring the ratio of succinic anhydride to lysine residue to 1:1. Again, 0.5 ml was withdrawn and marked "1:1." This procedure was continued for 2:1 and 10:1 molar ratios of succinic anhydride to lysine residue. After standing at room temperature for 30 min, the reaction mixture was passed over a column of Sephadex G-25 and was eluted with 0.1 M Tris-acetate buffer, pH 7.0.

Trinitrophenylation was carried out at room temperature by adding an aqueous solution of trinitrobenzene sulfonic acid to thyroglobulin in 0.1 M sodium bicarbonate solution, pH 8.0, in a manner similar to that described above for succinylation. The reaction product was passed over a Sephadex G-25 column to separate the protein derivative from the reagent. The protein derivative was eluted with 0.1 M Tris-acetate, pH 7.0, and the number of trinitrophenyl groups introduced per mol of thyroglobulin was determined from the absorbance (16) in neutral solution at 346 nm using an extinction coefficient of 14,500, or at 367 nm using an extinction coefficient of 10,500.

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Nitration of thyroglobulin was carried out at room temperature in 0.1 M sodium bicarbonate buffer, pH 8.0, by the addition of appropriate aliquots of 83 mM tetrinitromethane in ethanol. The reagent was added in such quantities as to make the ratio of tetrinitromethane to tyrosine the same as above, namely, 1:2, 1:1.2, and 1:1. The reaction mixture was allowed to stand at room temperature for 30 min prior to its application on a Sephadex G-25 column. The nitrotyrosylthyroglobulin was eluted with 0.1 M Tris-acetate buffer, pH 7.0. The number of nitrotyrosine groups was determined from the absorbance at 440 nm using an extinction coefficient of 4760 (17).

O-Acetylation of the tyrosyl residues of thyroglobulin was carried out in 0.1 M phosphate buffer, pH 7.0. N-Acetylimidazole (Sigma) was recrystallized from benzene. A benzene solution of acetylimidazole was prepared and the appropriate aliquot was placed in a test tube. The benzene was evaporated under a stream of nitrogen. 0.5 ml of thyroglobulin (17.5 mg/ml) was added to the acetylimidazole and the tube placed on a Vortex mixer to ensure thorough mixing. The reaction was allowed to remain at room temperature for 30 min prior to placing on a Sephadex G-25 column. The O-acetylated protein was eluted with 0.1 M phosphate buffer, pH 7.0. The number of tyrosyl groups acetylated was determined by mixing equal volumes of the modified protein and 2 M hydroxylamine, pH 7.5, and following the increase in absorbance at 278 nm as described by Riordan and Vallée (18).

Sedimentation velocity studies were carried out in the Spinco Model E analytical ultracentrifuge equipped with absorption scanner optics. The distribution of components of different sedimentation rates was estimated from the pen deflection after adequate resolution of the components had been achieved.

Ultraviolet absorption spectra and ultraviolet difference spectra were taken on the Cary recording spectrophotometer, Model 118C. Difference spectra in 5 M guanidine and in 1 M hydroxylamine, pH 7.5, and following the increase in absorbance at 278 nm as described previously (19).

Fluorescence spectra were taken with the Hitachi-Perkin Elmer spectrophotofluorometer, Model MPF44A, using microcuvettes. The cell compartment was maintained at 20 °C. The absorbance of all solutions was adjusted to 0.100 at 280 nm for fluorescence measurements.

Bovine thyroid membranes were prepared as described previously (20). Thyroglobulin and its derivatives (except succinyl thyroglobulin) were radio-iodinated using a lactoperoxidase procedure (12, 14). This method failed to label succinyl thyroglobulin which was successfully radiolabeled, however, using chloramine-T (20).

Assays to measure the binding of l-35S-labeled thyroglobulin and its derivatives to bovine thyroid membranes included the following: 20 μl of 0.1 M Tris-acetate buffer, pH 7.4, containing 2.5% bovine serum albumin; 10 μl of 0.2 M calcium chloride; 60 μg of bovine thyroid membrane contained in 10 μl; and 30 μl of water. The blank did not contain the membranes. In the studies on the time dependence of binding, the above assay mixture was incubated at the desired temperature for 15 min prior to the addition of 10 μl of 35S-labeled thyroglobulin containing approximately 100,000 cpm/assay. After the desired time period, the reaction was stopped by adding 2 ml of cold 0.025 M Tris-acetate buffer, pH 6.0, containing 1.25% bovine serum albumin, to the reaction tube and immediately pouring the mixture over a cellulose acetate filter (EHWP-02500, Millipore Corp.). The tube was rinsed another 2 times with 2 ml of the wash buffer. The filters were counted in the Beckman Model 9000 gamma counter. All assays were carried out in duplicate.

The protein concentration of native thyroglobulin was measured by absorption at 280 nm (ε1%1 cm = 10.0) (11).

RESULTS AND DISCUSSION

In the first part of this report, we will describe the physicochemical properties of the thyroglobulin derivatives in which the lysine residues were modified by succinylation or trinitrophenylation and the tyrosine residues were modified by reaction with tetrinitromethane or N-acetylimidazole. The binding of these derivatives to bovine thyroid membranes will be described in the latter portion of the paper.

Modification of Lysine Residues

Succinylation—Thyroglobulin (M, = 660,000) contains 130 lysine residues (2) whose side chains have a positively charged ε-amino group. Since charged lysine residues are predominantly on the "outside" of the protein, this amino acid side chain may interact with the thyroglobulin receptor of the membrane. In an attempt to determine whether this charged group is necessary for thyroglobulin to bind to the thyroid membrane, we succinylated the protein to different extents. The modification resulted in a negatively charged carboxylate group replacing the ε-amino function.

The effect of increasing the number of negatively charged succinate groups on the physicochemical properties of thyroglobulin is most readily demonstrated by an examination of the sedimentation velocity behavior of the modified protein (Fig. 1). The uppermost scan in this figure is thyroglobulin resulting from reaction mixtures containing 0.5 mol of succinic anhydride per mole of lysyl residues (0.5:1). The sedimentation coefficient of this material is 21 S which is slightly higher than the 19 S value of the starting material.

As the ratio of succinic anhydride to lysine decreases in the reaction mixture, the dissociation to 12 S thyroglobulin occurs. Thus, in the 1:1 succinylated derivative, 26% of the sedimenting material is 12 S; in 2:1 succinyl thyroglobulin, 60% of the protein is 12 S, and when the ratio of succinic anhydride to lysine is 1:0.1, all of the protein sediments with a homogeneous boundary having a sedimentation coefficient of 12 S. It is this 12 S material which will be used in further physicochemical studies and in the binding studies.

We were unable to detect a 17 S component formed as a result of succinylation, as had been reported by Edelhoch et al. (21).

In a previous study on the effect of succinylation of L-asparaginase on its physicochemical properties, Shifrin observed that extensive succinylation results in dissociation of the tetramer accompanied by extensive conformational changes of the monomer (22) which is reflected in spectrophotometric studies. The absorption maximum of a protein is generally shifted toward shorter wavelengths following dena-
there is a large, broad band with a maximum at the two prominent bands assigned to tyrosine and tryptophan, a result confirmed by ultraviolet difference spectroscopy (22). The resulting difference spectrum is shown by the dashed curve in Fig. 2. Yanari and Bovey (24) demonstrated that shifts to shorter wavelengths result when the chromophore is shifted from a region of low dielectric constant, such as would be found in the interior of a protein, to a region of high dielectric constant, such as the aqueous milieu on the outside of the protein. The peak in the region of 285 to 288 nm in the difference spectrum has been assigned to tyrosine residues (25, 26). In other words, when tyrosine is shifted from the hydrophobic interior of a protein to the aqueous environment of the medium, as would occur upon denaturation, peaks would be found in the region of 285 to 288 nm. The band with a maximum in the 292 to 294 nm region in the difference spectrum has been assigned to tryptophan (27). These bands could arise in the difference spectrum resulting from a comparison of 12 S succinyl thyroglobulin with native thyroglobulin if some tyrosines and tryptophans are located in the regions where the two 12 S subunits join to form the 19 S material. In addition, the 12 S polypeptide chain may be partially unfolded. For comparison, we examined the difference spectrum resulting from complete denaturation of unmodified thyroglobulin in 6 M guanidinium chloride with the spectrum of native thyroglobulin in buffer (Fig. 2). The difference spectrum below 330 nm is identical with the difference spectrum reported by Edelhoch and Metzger (28). In addition to the two prominent bands assigned to tyrosine and tryptophan, there is a large, broad band with a maximum at 313 nm. Since this band does not appear in other proteins, we tentatively assign it to the idotyrosines; this assignment is confirmed below. From the difference spectra we conclude that succinylation causes dissociation due to electrostatic repulsions, and results in the exposure of 25% of the tyrosines and tryptophans which can be exposed after complete dissociation by 6 M guanidine.

The emission maximum of tryptophan varies as a function of the dielectric constant of the solvent (29). For example, the emission of indole in n-hexane is 320 nm and in water it is 350 nm. L-Asparaginase from Escherichia coli contains one tryptophan residue per monomeric subunit and has an emission maximum at 320 nm; i.e., the medium in which the tryptophan residue is located has a dielectric constant equivalent to n-hexane. After exposure to 8 M urea, the tryptophan emission maximum is at 345 nm (19).

The fluorescence spectrum of native thyroglobulin (Fig. 3) shows a maximum at 327 nm in 0.1 M Tris-acetate buffer, pH 7.0. In the presence of 6 M guanidine, the intensity of the fluorescence increases and the maximum is shifted to 345 nm, as had been observed with many other proteins. If the succinyl thyroglobulin (10:1) were extensively unfolded, we would expect it to show an emission maximum at 345 nm. However, the emission maximum of the succinylated 12 S thyroglobulin is at 333 nm. These results are consistent with the observations from ultraviolet difference spectra since it indicates that some of the tryptophan side chains are exposed to an aqueous environment, but most of these aromatic residues remain in the interior of the protein in a medium of low dielectric constant.

Trinitrophenylation—The lysine residues were also modified by reaction with trinitrobenzene sulfonic acid (30). While this reaction results in the removal of the positive charge on the e-amino group of lysine and thereby changes the rate of migration in an electric field (31), it does not introduce a negative charge as occurred after succinylation. The same ratio of reagent to lysine were used here as were used with succinic anhydride. The moles of trinitrophenyllysine per mol of thyroglobulin were determined spectrophotometrically as described under "Materials and Methods" and are plotted as a function of the input ratio of 2,4,6-trinitrobenzene-1-sulfonate to lysine (Fig. 4).

The results indicate that at an input ratio of 1:1, 27 mol of lysine are trinitrophenlated and very few additional residues are modified even after the input ratio is increased to 10:1. Recent studies have indicated that the negative charge of the

![ULTRAVIOLET DIFFERENCE SPECTRA](image1)

![Fluorescence spectra of thyroglobulin and succinyl thyroglobulin](image2)
sialic acid residue interferes with the trinitrophenylation since 2,4,6-trinitrobenzene-1-sulfonate is negatively charged. Sedimentation velocity analysis indicates that the modified thyroglobulin is 19 S, i.e. that dissociation did not result as a consequence of modification of lysine residues.

It was not possible to study ultraviolet difference spectra as had been done with the succinylated derivative because of the large contribution of the trinitrophenyl moiety. Similarly, the nitrophenyl group quenches native fluorescence so that changes in protein conformation were not studied spectroscopically.

Modification of Tyrosyl Residues

Since the tyrosyl residues of thyroglobulin are the "active site" of iodination and thyroid hormone synthesis, the role of this aromatic group in maintaining the configuration of the iodoprotein has been studied extensively (32). In the present study, we have found that 20% of the tyrosyl residues ionize normally as determined spectrophotometrically (33), and that this same number of tyrosines are on the surface of the protein exposed to an aqueous environment, as determined by the solvent perturbation method of Herskovits and Laskowski (34). These studies confirm the values reported by Edelhoch (32). We have prepared several tyrosine derivatives in an effort to determine whether tyrosyl residues are involved in binding to thyroid membranes.

Nitration—Thyroglobulin was treated with increasing quantities of tetranitromethane in ethanol at pH 8.0. The number of moles of nitrotyrosine formed per mol of thyroglobulin was determined by the method described by Malan and Edelhoch (35) and is shown in Fig. 5. At the highest input of tetranitromethane/tyrosine (101), 40 nitrotyrosines are formed, or 33%. Since tetranitromethane has been reported to react with tyrosyl residues in an apolar environment in preference to exposed tyrosyl residues (36, 37), we are unable to determine how many of the exposed tyrosyl residues that we expect would be involved in binding to the membrane are nitrated.

One of the many side reactions resulting from the use of tetranitromethane is aggregation (38, 39). Sedimentation velocity analysis of the nitrated products of thyroglobulin indicated that they all had a homogeneous boundary with a sedimentation coefficient of 19 S; i.e. no significant aggregation occurred.

O-Acetylation—N-Acetylimidazole has been reported to acetylate only those tyrosyl residues that are exposed (40). In addition to the usual ratio of reagent:amino acid residue that we have used throughout this study (10:1), we have added a 60 molar excess to determine the maximum number of tyrosyl residues that can be acetylated. The number of tyrosyl residues that are acetylated was determined spectrophotometrically by following the increase in absorbance at 278 nm. A typical spectrum of O-acetyl thyroglobulin is shown by the dashed curve in the upper portion of Fig. 6, and the solid curve represents the spectrum of 1-acetyl thyroglobulin 30 min after being in the presence of 1 M hydroxylamine, pH 7.5. The spectral changes are more easily seen in the difference spectrum shown in the lower portion of the figure. In addition to the large band at 278 nm which is characteristic of the reappearance of the phenolic hydroxyl group, there is the reappearance of the band at 313 nm which was seen in the difference spectrum in guanidinium chloride (Fig. 2). This band can now be assigned to the iodothyrosine residues.

The number of tyrosyl residues that were acetylated as a function of the moles of N-acetylimidazole added per tyrosine residue is shown in Fig. 5. It has been suggested that N-acetylimidazole may be used to determine the number of "surface" tyrosyl residues in a protein (40, 41), i.e. the tyrosines that ionize with a normal pK of 10.5. In the case of thyroglobulin, however, there is a very large discrepancy between the tyrosines that ionize normally (28%) and those that can be acetylated with N-acetylimidazole (70%). In addition to reacting with tyrosine residues, the acetylimidazole can acetylate lysine residues (41); these are, however, not detected spectrophotometrically.

Sedimentation velocity analyses indicate that the O-acetylated thyroglobulin is 19 S, and the emission maximum at 327 nm indicates that the conformation of the acetylated protein remains intact.

Binding of Thyroglobulin Derivatives to Thyroid Membranes

The binding of 125I-labeled thyroglobulin and its derivatives to bovine thyroid membranes was carried out in 0.025 M Tris-acetate buffer, pH 7.0, containing 0.025 M calcium chloride and 0.6% bovine serum albumin. The results which will be presented were obtained at room temperature. The binding studies were also carried out at 37 °C, although at this higher...
Thyroid Receptor-Thyroglobulin Derivative Interactions

Fig. 6. Absorption spectra of O-acetyl thyroglobulin. Top: broken curve, absorption spectrum of 0.49 mg/ml of O-acetyl thyroglobulin in 0.1 M phosphate buffer, pH 7.0; solid curve, the same concentration of O-acetyl thyroglobulin after a 1-h exposure to 1 M hydroxylamine, pH 7.5 (final concentration). Bottom: difference spectrum obtained by subtracting the spectrum of 0.25 mg/ml of O-acetyl thyroglobulin in 0.1 M phosphate buffer, pH 7.0, from the spectrum of 0.25 mg/ml O-acetyl thyroglobulin after a 1-h exposure to a final concentration of 1 M hydroxylamine, pH 7.5. Tg, thyroglobulin.

with a high iodine content has a lower affinity for thyroid membranes than thyroglobulin with a low iodine content. In the present study, nitration of 33% of the tyrosine residues significantly reduces the affinity of thyroglobulin for the membrane, as shown by the binding curve in Fig. 7. Since we could not detect any other changes in the thyroglobulin molecule brought about by reaction with tetranitromethane, we can tentatively conclude that nitration of the tyrosine residues decreases the affinity for thyroid membranes.

The importance of the tyrosyl residue for binding to thyroid membranes is more readily demonstrated with the O-acetyltyrosine derivative of thyroglobulin, as shown by the bottom curve in Fig. 7 (solid triangles). Modification of 70% of the tyrosyl residues by O-acetylation dramatically reduces the ability of thyroglobulin to bind to thyroid membranes. Thus, after a 1-h incubation, only 2% of the added 125I-labeled acetyltirosine thyroglobulin was bound to the thyroid membrane. This decreased binding could be reversed by removing the O-acetyl group with hydroxylamine. The upper curve in Fig. 7B was obtained with O-acetyltyrosine thyroglobulin that was treated with 1 M hydroxylamine, pH 7.5, for 1 h and then dialyzed against 0.1 M Tris-acetate buffer, pH 7.0. The binding curve of the hydroxylamine-treated iodoprotein resembles the binding curves of lysyl-modified thyroglobulin. Studies of isoelectric focusing of this material suggest that the lysyl residues have also been acetylated by N-acetylimidazole.

In summary, we have demonstrated that modification of tyrosyl residues decreases binding of thyroglobulin to thyroid membranes, in particular, if the phenolic hydroxyl group is modified. We have also demonstrated that the state of aggregation of the iodoprotein does not appear to be an important factor, since both succinyl thyroglobulin (12 S) and trinitrophényl thyroglobulin (19 S) bind to the same extent. Our results also indicate that the positive charge on the lysine amino group is not directly involved in thyroglobulin-receptor interaction. In fact, modification of the lysyl residue may alter the configuration of thyroglobulin so that there is an increase in the number of residues important in the binding process. In this regard, the data in Fig. 7B are particularly notable, since modification of the tyrosine hydroxyl group eliminates the increased binding evident with lysine modification.

The results obtained in the accompanying report (14) showed that the N-acetylglucosamine residues on the B carbohydrate chains of thyroglobulin are also important in binding to thyroid membranes. Those observations indicated that there was a direct relationship between the exposed N-acetyleucosamine residues and binding to thyroid membranes, i.e. the greater the number of exposed N-acetylglucosamine residues, the greater the binding to the membrane. On the other hand, there was an inverse relationship between exposed N-acetylglucosamine residues and the iodotyrosine content of the thyroglobulin. The data were thus consistent with the idea that thyroglobulin molecules with exposed N-acetylglucosamine residues would be protected from degradation by binding to the thyroid membrane, but would be exposed to processes which result in the iodination of tyrosyl residues of thyroglobulin. The prediction that tyrosine residues might be a part of the membrane recognition process as well as the site of iodination is validated in this report.

Current studies are directed at questions concerning the relationship between N-acetylglucosamine and tyrosine determinants. Is there one receptor component which recognizes both the carbohydrate moiety and the amino acid side chain? Is there a relationship between the tyrosine recognition site and membrane-bound peroxidase?

It is evident that these studies concerned with the binding of thyroglobulin to membranes will provide new insights into...
thyroglobulin and its derivatives to thyroid membranes at room temperature, pH 7.0. A, O, 101 succinyl thyroglobulin; Δ, 101 TNP thyroglobulin; □, unmodified thyroglobulin; ◊, 101 nitrothyroglobulin; ▲, 101 O-acetyl thyroglobulin. B, solid curve, 101 O-acetyl thyroglobulin; broken curve, 101 O-acetyl thyroglobulin after removal of the acetyl group with 1 M hydroxylamine, pH 7.5. The concentration of membranes was 50 μg. TNP, trinitrophenyl.

the structural features of the thyroglobulin molecule which are important for post-translational processing and vital to the formation of thyroid hormones.

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