The Properties of Thyroglobulin

IX. THE MOLECULAR PROPERTIES OF IODINATED THYROGLOBULIN

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Iodine reacts readily with many proteins and has been used as a group-specific reagent to study the relationship between enzyme and group activity (see, for instance, (1, 2)) (3). Iodoprotein derivatives have also been prepared as electron dense protein crystals for use in x-ray investigations (4). At very low levels of iodination, radioactively labeled iodoproteins have been employed as tracers in lifetime studies of serum proteins (5, 6) and in other biological processes (7, 8).

Although many proteins have been iodinated and used as investigative tools, few have been extensively characterized in terms of their physicochemical behavior. In a careful study of human serum albumin, Hughes and Strassle (4) were able to crystallize iodine derivatives containing up to 15 atoms of iodine per mole of albumin. In an investigation of the mobility distributions of several iodinated preparations of human serum albumin by electrophoretic boundary spreading experiments, Baldwin, Laughton, and Alberty (9) found that substitution of 7.2 iodine atoms per mole had only a relatively small effect on the mobility distribution. A considerable increase in heterogeneity was observed when 18 iodine atoms were introduced into the protein. At 40.5 atoms, the mobility distribution was very broad and apparently only 50% of the protein was soluble.

In the accompanying report (10), the distribution of iodine in tyrosine and thyronine derivatives in native and iodinated thyroglobulins has been evaluated by spectrophotometric methods. The effects of iodination on the molecular properties of thyroglobulin have been studied by a variety of methods which depend on various aspects of its macromolecular structure and are reported herewith.

EXPERIMENTAL PROCEDURE

Iodimetry—Sodium thiosulfate (0.05 M) solutions were prepared by weight from the pentahydrate salt and standardized against a KMnO₄ solution. KMnO₄ (0.05 M) and As₂O₃ (0.05 M) solutions were prepared by weight from dried reagents. Iodine solutions contained 12 moles of KI per mole of iodine and were stored in dark bottles. The molarity of iodine solutions was determined by titrating with standard sodium thiosulfate solutions in HCl with starch as an indicator. Dioxide thiosulfate solutions are unstable and were therefore prepared fresh from the concentrated stock. As₂O₃ was standardized with iodine solution at pH 8.3 in Tris buffer with starch used as an indicator.

Iodination—Iodine solution (0.04 M) in 0.48 M KI was added at a constant rate through a fine polyethylene catheter to a 0.8% thyroglobulin solution in 0.16 M glycine. The solution was stirred (magnetically) throughout, and the pH was maintained at 9.0 by a pH-Stat. Varying amounts of KI were added after iodination in order to bring all solutions to 0.057 M so as to compensate for the iodide formed by the substitution reaction. Solutions were kept at room temperatures until the iodine color disappeared and were then stored at 3°. Thyroglobulin solutions iodinated with 85 and 110 moles of iodine were colorless. Those iodinated at higher levels showed increasing yellow color.

Kinetic experiments were performed at constant pH (pH-Stat) at a controlled temperature. Aliquots were removed and rapidly delivered into an equal volume of 1 M HCl to stop the reaction. The unchanged iodine was determined by titrating with standardized sodium thiosulfate.

Spectrortitrations—The spectrometric titration of native and iodinated thyroglobulins is described in the accompanying report (10). Forward and reverse curves were obtained by adding small volumes of 2.0 M KOH or HCl, respectively, to protein solutions.

Protein Preparations—Thyroglobulin was prepared by a differential centrifugation procedure, which is described elsewhere (11). The preparations used in this study contained 95% S-19 thyroglobulin and 5% of a faster sedimenting component. The preparation of rabbit antithyroglobulin antibodies is reported in a recent communication (12). The procedure used to measure the precipitin curve was identical with that employed to determine the inhibitory activity of thyroglobulin fragments in the homologous antigen-antibody reaction (13). Bovine serum albumin was a crystalline product of the Armour Labora
tories, Kankakee, Illinois. Bovine pancreas ribonuclease was 5 times crystallized and obtained from Sigma Chemical Company, St. Louis. Protein concentrations were determined from their absorption at 280 mμ at neutral pH. The extinction coefficients employed for a 1.0% solution in a 1.00 cm² cell of thyroglobulin, bovine serum albumin, and ribonuclease were 10.5, 6.6, and 6.95, respectively.

Physicochemical Measurements—The viscosity determinations were made at 25.0°. Optical rotation measurements were made in a Rudolph polarimeter, model 80. The procedures used in these sedimentation and denaturation measurements are described in earlier reports on thyroglobulin. In the experiments on the effect of alkali on the sedimentation properties of thyroglobulin, the solutions were sedimented immediately after increasing the pH to the final value. Relative amounts of components were obtained from the areas under their boundaries.
No corrections were made for either the radial dilution of the solute or the Johnston-Ogston effect.

**Enzymatic Hydrolysis**—The kinetics of tryptic hydrolysis of thyroglobulin were followed by an automatic recording pH-Stat by utilizing the radiometer TTT1 pH meter, Ole Dihc recorder, and Agla precision syringe. Nitrogen, which was passed through a concentrated NaOH solution to remove CO2, was blown over the solution. The reaction was initiated by adding a very small volume of trypsin after the protein solution was equilibrated until the pH ceased to drift. At the reaction pH (8.00), the α-amino groups that are formed by proteolysis will release part of their protons. The pH-Stat operates by neutralizing the acid released by adding just enough base to maintain the pH constant.

**RESULTS**

**Rates of Iodination**—The influence of pH on the rate of disappearance of iodine in thyroglobulin solutions is shown in Fig. 1. It is apparent that the rate increases rapidly with pH in accord with Li’s findings on the rate of iodination of tyrosine (14). Since the iodine concentration was 2.50 × 10⁻⁴ M, an iodine to protein molar ratio of 193 resulted. Spectral analysis of the intact protein gave a tyrosine value close to 106 (10). Since only approximately 70% of the tyrosine residues are iodinated in aqueous media, the concentration of iodine was in excess of stoichiometric for the reactive groups (10).

The rates of iodination of two other proteins, bovine serum albumin and ribonuclease, have been compared with thyroglobulin and are shown in Fig. 2. The protein concentrations were adjusted so that their solutions contained 1.25 × 10⁻⁴ mole of tyrosine residues per liter. Since the iodine was 2.50 × 10⁻⁴ M, enough iodine was present to convert all of the tyrosyl groups to diiodotyrosine. As seen in Fig. 2, thyroglobulin and ribonuclease react with iodine at closely similar rates at pH 9.10; however, they react considerably more slowly than bovine serum albumin. At pH 7.02, although all rates were reduced significantly, the relative rates of iodination of the three proteins remained similar. In 8 M urea at pH 9.1, the iodine completely disappeared in less than 2 minutes (Fig. 2). This very rapid reaction with iodine may arise from various properties of the system. (a) Li has shown that the rate of iodination of tyrosine increases with urea concentration (15); (b) in concentrated urea solutions the phenolic hydroxyl groups of thyroglobulin are normalized and react almost quantitatively with iodine (11); and (c) part of the iodine that disappears may be accounted for in a very rapid reaction with sulfhydryl groups which become accessible in concentrated urea solutions.

**Spectrophotometric Titration of Phenolic Groups**—Native thyroglobulin: it has been reported that the ionization of the tyrosyl groups in native thyroglobulin is displaced toward more acid values on back-titration from above pH 12 (16). The onset of irreversibility in the titration of these groups was determined by initiating the reverse titration at a series of pH values. The data of four such titrations are illustrated in Fig. 3. It can be seen that the degree of hysteresis increased as the pH of initiation of the reverse titration was increased. When the vertical displacement in absorbancy between the forward and reverse curves at pH 11.0 was plotted against the pH of reversal, an approximately linear relation was found (inset, Fig. 3). Extrapolation of this curve to zero displacement indicates the pH at which the tyrosyl groups no longer ionize reversibly. A pH value of 11.05 was obtained in this way. This value is slightly below the pH value at which the denaturation of thyroglobulin occurs at a measurable rate.

Iodinated thyroglobulin: the titration curve of thyroglobulin treated with 110 moles of I2 in aqueous media is shown in Fig. 4. A distinctive plateau region is evident in the pH range 8.5 to 10, which serves to separate the diiodotyrosyl and tyrosyl ionizations and approximately demarcates the range of monoiodotyrosyl ionization. On back-titration from pH 12.4, the reverse curve formed a closed hysteresis loop in the pH region in which the tyrosyl residues were predominantly titrated.
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FIG. 3. Spectrotitration curves at 300 mμ of native thyroglobulin. ●, Forward curve. Reverse curves: ■, from pH 11.2; ▲, pH 11.4; ▲, pH 11.6; ○, pH 12.5; inset, change in absorbancy between forward and reverse curves at pH 11.0 as a function of the pH of reversal. Solutions contained 0.300% protein, 0.15 M KCl, and 0.015 M lysine.

FORWARD

REVERSE FROM 12.5

REVERSE FROM 9.5

PH

FIG. 4. Spectrotitration at 305 mμ of thyroglobulin iodinated with 110 moles of iodine. Solution contained 0.115% protein, 0.028 M glycine, 0.028 M lysine, and 0.15 M KCl. Temperature was 2.5°C. Iodination and titration were in aqueous solution.

The forward and reverse curves coincided when back-titration was begun at pH 9.5. It should be noted here that at this level of iodination almost all of the properties of the molecule closely resemble those of native thyroglobulin (see below). At 55 moles of I₂, completely analogous results were obtained in that the iodinated amino acid residues titrated reversibly, whereas the tyrosyl residues formed a closed loop between pH ~9 and 12.4.

When thyroglobulin was treated with 220 moles of I₂ in water, its molecular properties corresponded rather closely with that observed for its thermally denatured form (see below). As illustrated in Fig. 5, the iodinated groups ionize reversibly, whereas the uniodinated tyrosyl residues show approximately the same behavior as that found in the native form of the protein. It should be recalled, however, that most of the tyrosyl residues in thermally denatured thyroglobulin also titrate abnormally (16).

When thyroglobulin was iodinated in 8 M urea, the substitution of iodine was more extensive. The forward and reverse titration curves of thyroglobulin iodinated in 8 M urea with 110 mole of I₂ appear in Fig. 6 (the titration being performed in 1 M urea). It can be seen that the diiodotyrosyl and tyrosyl ionization regions are no longer separated by a plateau region. As pointed out in the accompanying paper (10), there is a considerable increase in monoiodotyrosine at this level of iodination, which occurs in 8 M urea but not in aqueous media. The back-titration curve from pH 12.45 shows a hysteresis loop that is considerably shallower than that observed in the aqueous titrations, whereas reversal from pH 9.0 retraced the forward curve. The behavior of thyroglobulin iodinated with 55 moles of iodine in 8 M urea was analogous to that reported for the 110 moles of I₂ level, although fewer groups were iodinated.

When thyroglobulin that had reacted with 220 moles of I₂ in 8 M urea was titrated, the hysteresis effect noted at lower levels of iodination disappeared. The titration curve, as seen in Fig. 6, was completely reversible from pH 12.45. It is also evident from the small absorbancy change that occurs above pH 10 that very few uniodinated tyrosyl residues are left.

Molecular Properties—Remarkably little change occurred in

FIG. 5. Spectrotitration at 305 mμ of thyroglobulin iodinated with 220 moles of iodine. Other conditions were as stated in the legend to Fig. 4.

FIG. 6. Spectrotitration at 305 mμ of thyroglobulin iodinated with 110 moles (right hand curve) and 220 moles (left hand curve) of iodine in 8.0 M urea. Solutions contained 0.10% protein, 0.025 M glycine, 0.050 M lysine, 0.13 M KCl, and 1 M urea.
iodinated with either 55 or 110 moles of iodine in aqueous and denaturation was close to the final value, whereas the rota-

 graded S-19. As may be seen in Fig. 7, the change in viscosity in roughly equal amounts, replaced the S-19 peak of the native protein in the velocity sedimentation pattern. These peaks have been identified from their sedimentation coefficients as the S-12 and S-17 components, normally found in thermally de-

 natured solutions, and S-9, the principal component of alkali-
denatured thyroglobulin.

At 220 moles of iodination, the sedimentation diagram showed four boundaries, the above three and a small amount of unde-

 grated S-19. As may be seen in Fig. 7, the change in viscosity and denaturation was close to the final value, whereas the rotation was considerably less than found with 350 moles. After iodination with 290 moles of iodine, most of the total change had occurred also in the specific rotation.4

Tryptic Hydrolysis—The rate of proteolysis of thyroglobulin at pH 8.0 at 30.0° was followed until the pH-Stat showed no change in a 2 minute interval. The continuous rate curves obtained on the recorder for thyroglobulin treated with either 55 or 110 moles of iodine were indistinguishable from those for the native untreated protein. A marked increase in rate and amount of acid liberated was observed, however, at 220 moles and at higher levels of iodine.

The data have been interpreted in terms of two simultaneous apparent first order reactions. This type of analysis has been applied to the fibrous proteins, myosin (17) and collagen (18), with quite interesting results. The total number of groups hy-

 drolized (A∞) in the two first order reactions was obtained by extrapolating to zero rate a plot of the rate of change in volume of base against the net volume of base added in the pH-Stat. A first order plot of the acid liberated in the hydrolysis of thyro-

globulin, iodinated with 350 moles of iodine, is shown by the points in the upper curve in Fig. 8. The velocity constant of the slower reaction was obtained from the slope of the line drawn through the points obtained after approximately 4 minutes of reaction time. The number of peptide bonds hydrolyzed by the second (slower) phase was obtained from the extrapolated value of the straight line to the intercept with the ordinate. When the extrapolated values of the slower phase were sub-

 tracted from the initial portions of the plot, a linear relation was also found for the first or rapid phase of the reaction (lower curve, Fig. 8). The velocity constant of this phase was approxi-

 mately 3 times faster than that of the slow phase (Table I).

If the pK of the amino group formed by hydrolysis is assumed to be 7.83, then each mole of base consumed represents 60% of the total groups hydrolyzed at pH 8.0. Since 159 moles of base were consumed, the total number of bonds hydrolyzed was 265. This represents only 60% of the potentially available groups, since there are 130 moles of lysine and 322 moles of arginine residues per mole of thyroglobulin5 (molecular weight, 670,000).

Table I

<table>
<thead>
<tr>
<th>Bonds cleaved</th>
<th>k (fast)</th>
<th>k (slow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>min⁻¹</td>
</tr>
<tr>
<td>110</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>350</td>
<td>20</td>
<td>38</td>
</tr>
</tbody>
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4 Since all of the iodine was consumed in these solutions and not much increase occurred in the degree of substitution into tyrosyl residues at levels of iodine above 220 moles, it appears that the oxidative side reactions that occur above this latter level of iodine have very little effect on the configurational properties of (denatured) thyroglobulin other than the final small increase in specific rotation.

5 Obtained by K. Piez by column chromatography on highly purified calf thyroglobulin. This preparation showed only one boundary in the ultracentrifuge when examined at 1.5% protein concentration.
MOLES IODINE

MINUTES

FIG. 9. Rate of thermal fragmentation at 46.0° of the S-19 component in native and lightly iodinated thyroglobulins. Solutions contained 0.70% protein, 0.003 M KNO₃, 0.015 M KI, and 0.01 M glycine buffer at pH 9.6. S-12 component: ●, 55 moles of iodine; ○, 110 moles of iodine. S-17 component: ■, 55 moles of iodine; ▲, 110 moles of iodine. Experiments were performed at room temperatures.

FIG. 10. Effect of alkali on the disappearance of the S-19 component in native and lightly iodinated thyroglobulins. Solutions contained 0.70% protein, 0.10 M KNO₃, 0.015 M KI, 0.01 M glycine, and 0.012 M lysine.

The data on the native thyroglobulin, or on the protein treated with 110 moles of iodine, fit equally well into the kinetic analysis described above for the iodine-denatured thyroglobulin. The total number of groups hydrolyzed, however, was only 26% of the total available. Nevertheless, it is of interest that the rate constants for the fast and slow phases were almost identical with those observed with the highly iodinated molecule. The number of groups hydrolyzed and their respective rate constants are summarized in Table I. The net effect appears to be an approximate doubling of the number of bonds hydrolyzed in each phase.

Since the mean sedimentation coefficient of a slowly moving boundary seen in trypsin-digested thyroglobulin continues to fall during a period of 24 hours (13), it is evident that at least a third set of groups is hydrolyzed with a markedly smaller rate constant than the two considered in the above kinetic analysis.

It should be stated that the different phases of hydrolysis alluded to above probably do not represent the rate of hydrolysis of a single type of peptide linkage, but rather a group of bonds with similar rate constants.

Heterogeneity—In an earlier report (16), data were presented indicating that, although purified native thyroglobulin showed a single, symmetrical boundary on ultracentrifugation, it did not behave as a single species when subjected to either thermal or alkaline stress. The extent of disappearance of native protein (S-19) was governed by the temperature or pH of the reaction. Since the iodine content in thyroglobulin is known to vary, whereas the amino acid composition (exclusive of tyrosine and its iodinated derivatives) appears to be constant (19), it was suggested that the failure of native thyroglobulin to act as a single species was based on a heterogeneous distribution of iodine among the thyroglobulin molecules. Further data in support of this thesis are presented in Figs. 9 and 10. The rate and extent of fragmentation of S-19 thyroglobulin into S-12 and S-17 components at 46° (pH 9.6) are seen to be markedly dependent on the degree of iodination. Similar differences in stability between native and partially iodinated thyroglobulin were observed in alkaline solutions (Fig. 10). Increasing iodination shifts the pH profile curve to lower pH values.

When the S-12 component is formed from native thyroglobulin, it is almost always present in greater amounts than the S-17 (11, 20). However, in the iodinated preparations studied between pH 9.5 and 11.4 (Fig. 11), the amount of S-12 formed at a specific pH remained constant, whereas the S-17 component increased with the extent of iodination. In thyroglobulin that had reacted with 110 moles of iodine, the S-17 particle became the principal product when the pH was increased to 11.4.

Comparable effects were evident also in the thermal breakdown of iodinated thyroglobulin. Similar amounts of S-12 and S-17 components were formed in thyroglobulin iodinated with 55 moles of iodine (Fig. 9). At twice this level of iodination, the S-12 unit increased only marginally, whereas the S-17 approximately doubled. The significance of this altered pattern of behavior of iodinated thyroglobulin is not apparent at present.

Antigenic Properties—In order to characterize iodinated thy-
slower sedimenting components cross-react with antibodies formed against native (S-19) thyroglobulin. It is evident that the properties of highly iodinated and thermally denatured thyroglobulin bear a notable resemblance to each other. In fact, even the rates of tryptic hydrolysis are very similar. This identification, however, is not supported by all of the data. The sedimenting components observed in highly iodinated thyroglobulin reveal an S-9 boundary that is typical of alkali and not of thermal denaturation. As reported elsewhere (11), when thyroglobulin is denatured by alkali, the S-12 and S-17 components; the greater the degree of iodination, the more pronounced the effect. It has been proposed previously that the heterogeneity in stability observed at either high temperatures or alkaline pH values arise from an unequal distribution of iodine in native thyroglobulin molecules. The data on iodinated thyroglobulin, which still retains all of its normal configurational parameters, furnish direct support for this concept. It is also evident that the breakpoint of S-19 thyroglobulin into slower sedimenting components is indeed a most sensitive measure of structural alteration.

It is evident that the behavior of their phenolic hydroxyl groups is different from that of thyroglobulin. In bovine albumin, all of the groups ionize reversibly but with a single, slightly elevated pK and a markedly higher heat of dissociation (21).6 Ribonuclease has two distinctive pH ranges of tyrosyl ionization. Three of the six groups ionize with normal constants, and three appear only when the native structure is destroyed by alkali above pH 12.5 (22). The ionization behavior of the tyrosyl groups in thyroglobulin cannot be unambiguously determined from titration data, since configurational changes are known to overlap their ionization range and will lead to a modified titration curve. Only approximately 20% of the tyrosyl residues dissociate before the structural modifications, which commence near pH 11.0 and alter the behavior of the remaining groups.

Cha and Schoraga (23) have recently reported that four of the six tyrosyl groups in ribonuclease may be converted to diodo-tyrosine in aqueous media and that two are refractory to iodination (23). In the accompanying report (10), it is shown that approximately one-third of the tyrosyl groups in thyroglobulin are unreactive even in excess iodine. Thus, despite the different stability and pattern of tyrosyl ionization observed in these two globular proteins, their rates and extent of substitution appear to be comparable. In bovine albumin, iodination proceeds much more rapidly and may be associated with the reversibility of the phenolic hydroxyl ionization. It should be noted, however, that when stoichiometric amounts of iodine are used, Li and Hughes and Strasassel (4) estimate, by quite different procedures from those used above, that approximately 50% and 30% of the tyrosyl groups remain uniodinated in serum albumin.

The most striking result of the molecular studies is that 75 atoms of iodine may be substituted in tyrosyl groups in thyroglobulin (on addition of 110 moles) with almost no discernible effect on its configuration. Even its fine structure appears to be unaffected, since the rate of tryptic digestion, which one would presume to be very sensitive to minor structural modifications, is unchanged from that of the native molecule. It is unlikely that two compensating effects on rate balance each other, since no effect on the rate of proteolysis is observed when thyroglobulin is iodinated with either 55 or 110 moles of iodine.

Of all of the molecular variables studied, only the fragmentation studies were able to resolve a significant difference in behavior between native thyroglobulin and thyroglobulin treated with either 55 or 110 moles of iodine. Both the thermal and alkaline pH treatments resulted in an enhanced formation of S-12 and S-17 components; the greater the degree of iodination, the more pronounced the effect. It has been proposed previously that the heterogeneity in stability observed at either high temperatures or alkaline pH values arise from an unequal distribution of iodine in native thyroglobulin molecules. The data on iodinated thyroglobulin, which still retains all of its normal configurational parameters, furnish direct support for this concept. It is also evident that the break-up of S-19 thyroglobulin into slower sedimenting components is indeed a most sensitive measure of structural alteration.

6 C. Tanford has informed the authors that these values do not necessarily characterize the behavior of the tyrosyl groups in serum albumin, since the molecular transition that occurs in this pH range may considerably affect these values.

7 H. Edelhoch, unpublished data.
2. Only above ~40% iodination of the tyrosyl groups do the configurational properties of thyroglobulin change. At higher levels of iodination, thyroglobulin becomes denatured, and its properties closely resemble those of its thermally denatured form. Further molecular changes occur in alkaline solution, as shown by the hysteresis effects in the behavior of the unchanged tyrosyl groups.

3. In partially or maximally iodinated thyroglobulin, the iodinated tyrosyl groups ionize reversibly.

4. No difference has been found in the rate of tryptic hydrolysis between native and lightly iodinated thyroglobulin.

5. The only important difference found between native thyroglobulin and thyroglobulin iodinated with less than 110 moles of iodine is in the susceptibility of the iodinated forms to either thermal or alkaline fragmentation.

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