Relationship between Iodination and the Polypeptide Chain Composition of Thyroglobulin

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Thyroglobulin was isolated from thyroid glands of normal guinea pigs and from animals treated with thiouracil. These preparations were fractionated by isopyknic centrifugation in RbCl into proteins of varying iodine content. When the disulfide bonds of these protein fractions were reduced and analyzed by polyacrylamide gel electrophoresis in Na dodecyl-SO₄, three species were observed with molecular weights of 295,000 (A), 210,000 (B), and 110,000 (C). Species A comprised 80% of the protein in thyroglobulin of 0.04% iodine and 13% in thyroglobulin of 0.68% iodine content. Species C showed the opposite relationship, comprising 10% of the low and 70% of the high iodine thyroglobulin. Species B was relatively independent of the iodine content and represented approximately 20% of the protein. Iodine analysis of these proteins showed species A to be lowest and species C highest. It appears that the subunit composition of thyroglobulin depends on the degree of iodination and that species A should be the only one present in the absence of iodination.

The polypeptide chain composition of guinea pig thyroglobulin (19 S) contrasts significantly with that of thyroglobulin isolated from other species, such as hog, sheep, bovine, human, and rat. In the latter species, when reduced and alkylated thyroglobulin is analyzed by hydrodynamic methods, conflicting results have been obtained on the number and size of the elementary polypeptide chains (1-3). A considerably higher degree of size heterogeneity is observed when these preparations are examined by polyacrylamide gel electrophoresis in Na dodecyl-SO₄ where apparent molecular weights ranging from 15,000 up to 330,000 (4-6) were observed. It has been recently demonstrated that with highly purified guinea pig thyroglobulin, after complete reduction of the S-S bridges and alkylation of the resulting —SH groups, only three distinct groups of bands are found when analyzed by polyacrylamide gel electrophoresis in Na dodecyl-SO₄ (7). The large number of small size chains (<100,000) were missing in the guinea pig preparations. Three species were also obtained with reduced and alkylated protein by gel filtration in 6 M Gdm-Cl and shown to have molecular weights of approximately 235,000, 210,000, and 110,000 by equilibrium sedimentation in 6 M Gdm-Cl (7).

Although the guinea pig system seems to be simpler than that of the other species studied, it is important to understand the origin of the three polypeptide fractions observed by electrophoretic analysis. Such heterogeneity could be related either to different genes coding for the different polypeptide chains or, alternatively, to post-translational modifications occurring after the synthesis of the elementary chains. Since iodination is known to affect various properties of 19 S thyroglobulin (8-10), an attempt has been made to correlate the polypeptide chain composition with the iodine content of guinea pig thyroglobulin. Thyroglobulin preparations of widely different iodine content have been obtained from an inbred strain of guinea pig, fractionated by isopyknic equilibrium centrifugation in RbCl according to their iodine content (11, 12), and analyzed by gel electrophoresis. A definite dependence of the relative amounts of the three polypeptide fractions on iodine content has been demonstrated.

EXPERIMENTAL PROCEDURES

Materials

Male guinea pigs, National Institutes of Health strain, inbred, weighing 200 to 250 g were used. All animals were fed regular guinea pig diet supplemented with vitamin C. Optical grade RbCl was used (Schwarz/Mann). All other chemicals were reagent grade.

Methods

Preparation of Thyroid Iodoproteins—Three preparations of thyroglobulin of different iodine content were obtained: (a) from normal animals (0.47% average iodine content); (b) from animals fed with regular guinea pig diet supplemented with 0.02% thiourea (w/w) for 21 days (0.2% average iodine content); and (c) from animals on a similar regimen for 60 days (0.65% average iodine content).

After removal of the thyroid glands, extraction with buffer (0.1 M sodium phosphate, pH 7.2), and ammonium sulfate precipitation (1.4 M to 1.8 M), the 19 S thyroglobulin and the other naturally occurring thyroid protein, i.e. 27 S, were isolated by gel filtration on Bio-Gel
phoresis in Na dodecyl-SO₄—Since Na dodecyl-SO₄ precipitates in 0.0241 sodium azide, and extensively dialyzed against 0.05 M sodium phosphate, pH 7.2, the two proteins, the molecular weights of which differ by a factor of 2, were almost completely resolved by this procedure.

Preparation of Samples for Analysis on Polyacrylamide Gel Electrophoresis in Na Dodecyl-SO₄—Since Na dodecyl-SO₄ precipitates in RbCl, it was necessary to remove RbCl by dialysis before addition of Na dodecyl-SO₄. Na dodecyl-SO₄ was then added to a final concentration of 1% and reduction of the disulfide bonds was accomplished with 2-mercaptoethanol (5%) by incubating for 4 hours at 27° at pH 7.2. The efficiency of this reduction procedure is comparable to reduction in 6 M Gdm-Cl, as has been described elsewhere (7).

Polyacrylamide Gel Electrophoresis in Na Dodecyl-SO₄—The conditions of electrophoresis, staining and destaining of the gels, have been described elsewhere (7). Each Coomassie blue R-250-stained gel was scanned at 550 nm with a Gilford gel scanner after destaining in 5% methanol and 7% acetic acid. The relative amounts of the components were obtained by graphical integration of the various peaks. Scans of gels with the same protein sample at different concentrations were performed and found to give a linear dependence of protein concentration on absorbance.

Analytical Procedures—The protein concentration was measured by absorption at 280 nm using an extinction coefficient (εₒ) of 10.0. Iodine analysis was performed by Boston Medical Laboratories using a modified Zak procedure (13).

RESULTS

Fractionation of Normal Guinea Pig Thyroglobulin—Ultracentrifugally homogeneous preparations of 19 S thyroglobulin (44 mg) and 27 S iodoprotein (4.9 mg) were obtained by gel filtration of Bio-Gel A-5m from a single pool of 20 normal guinea pig thyroids. Both iodoproteins were fractionated by preparative isopyknic centrifugation in RbCl (Fig. 1). The 27 S iodoprotein is more narrowly distributed than thyroglobulin and significantly shifted toward the higher density regions of the gradient.

The isopyknic fractionation of the 19 S and 27 S iodoproteins was demonstrated by analyzing various parts of the gradient for iodine content. The 19 S was divided into five portions and the 27 S into two. The results of the iodine analyses are reported in Table I. The iodine content progressively increases from the lower to the higher density regions of the gradients, with the exception of the last two fractions which have similar iodine content. It has been observed in several experiments of this kind with different thyroglobulin preparations that the iodine content in the descending part of the gradient varied much less than in the ascending half and frequently the variation in iodine between the last two denser fractions was quite small. It appears that the pool of 19 S protein in the follicular lumen is not randomly iodinated, since a Gaussian distribution of iodine would be expected if the iodination process was completely unrelated to the level of iodination, unless the level of iodination is approaching saturation. It should also be noted that the 27 S iodoprotein differs from 19 S thyroglobulin only in lacking the less iodinated species (Fig. 1).

Subunits of Thyroglobulin—The various thyroglobulin fractions obtained by RbCl isopyknic centrifugation were analyzed by polyacrylamide gel electrophoresis in Na dodecyl-SO₄, before and after reduction. It is known from ultracentrifuge studies that only 12 S is formed from 19 S by denaturating conditions in the absence of reduction and that 19 S is incompletely dissociated (1, 10). In unreduced 19 S, the two bands present in all fractions, correspond to molecular weights of 660 x 10⁶ and 330 x 10⁶ and represent more than 95% of the total protein stained on the gel (Fig. 2). The relative amounts of the two bands, however, change as a function of the iodine content of each sample. In Fig. 3, the percentage of 12 S

![Fig. 1. Distribution pattern of protein of guinea pig 19 S (■—■) and 27 S (■—■) after equilibrium centrifugation in RbCl.](http://www.jbc.org/)

**FIG. 1.** Distribution pattern of protein of guinea pig 19 S ( ■—■ ) and 27 S ( ■—■ ) after equilibrium centrifugation in RbCl. The protein was measured by absorption at 280 nm. The tubes that were combined to form a single sample are given in Table I.

**TABLE I**

<table>
<thead>
<tr>
<th>Fract.</th>
<th>Iodine content (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a (8-11)</td>
<td>0.28</td>
</tr>
<tr>
<td>b (12-14)</td>
<td>0.35</td>
</tr>
<tr>
<td>c (15-19)</td>
<td>0.46</td>
</tr>
<tr>
<td>d (20-23)</td>
<td>0.62</td>
</tr>
<tr>
<td>e (24-27)</td>
<td>0.63</td>
</tr>
<tr>
<td>f (8-11)</td>
<td>0.04</td>
</tr>
<tr>
<td>g (12-15)</td>
<td>0.06</td>
</tr>
<tr>
<td>h (16-19)</td>
<td>0.16</td>
</tr>
<tr>
<td>i (20-24)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*Tube numbers of the gradient (see Fig. 1).
*Thyroglobulin preparation from animals treated with 0.02% thiouracil for 60 days (see "Methods").
FIG. 2. Electrophoretic analysis of the subunit composition of native 19 S iodoprotein of different iodine contents and native 27 S. Electrophoresis on Na dodecyl-SO₄-polyacrylamide gels of 19 S fractions a, c, and e, obtained from RbCl centrifugation (see Fig. 1 and Table I) and 27 S (unfractionated). The total gel concentration and cross-linking were 4%. Electrophoresis lasted 3½ hours at 8 mA/tube. The gel was buffered with 0.1 M sodium phosphate, pH 7.1, and contained 0.1% Na dodecyl-SO₄ and 0.02% sodium azide. The slower and faster migrating bands in the 19 S gels represent 19 S and 12 S, respectively (as indicated). It should be noted that the concentrations of protein were not the same in all of the gels.

subunits is plotted against the iodine content of thyroglobulin. It is evident that, within the range of iodine content now examined (0.28 to 0.69% iodine), there is a direct relationship between iodination level and the fraction of subunits which are covalently linked.

The preparation of guinea pig 27 S having an iodine content of 0.55% was also analyzed by gel electrophoresis in Na dodecyl-SO₄. Less than 20% of 27 S dissociated into 12 S subunits. At a comparable level of iodination, approximately 40% of 19 S dissociated into 12 S.

Polypeptide Chains of Thyroglobulin—Electrophoresis in Na dodecyl-SO₄ gels of reduced guinea pig thyroglobulin demonstrated the presence of three molecular species which taken together represented more than 95% of the protein stained with Coomassie blue (Fig. 4). As previously reported (7) each species consists of a major and one or two closely migrating satellite bands. The molecular weights of these three species were shown previously to correspond to 295, 210, and 110 x 10⁶ by sedimentation equilibrium in 6 M Gdm-Cl (7). When the fractions obtained by equilibrium centrifugation in RbCl were reduced and analyzed by gel electrophoresis, a clear dependence of the relative amounts of the three species on the iodine content was evident. The relative amounts of the three species in the thyroglobulin fractions isolated from the same 19 S preparation are shown in Fig. 5. The percentage of the largest species (A) is inversely related to the iodine content of thyroglobulin, whereas the percentage of the smallest species (C) is directly related to the iodine level. The intermediate species (210,000) remained nearly constant at 20% of the total protein. In the 27 S iodoprotein with 0.55% iodine, the proportion of the smallest species was close to that observed in the more highly iodinated 19 S fraction (Fig. 4).

Since the relative amounts of the three major species vary with the iodine level between 0.25 and 0.70, it was of interest to see if this relationship extended to very low iodine levels. Low iodine preparations of 19 S were therefore obtained from guinea pigs treated with thiouracil and fractionated by RbCl centrifugation. The least dense fraction was found to contain 0.04% iodine (2 atoms/mol of 19 S) and was compared with the iodinated fractions obtained from untreated animals.

Electrophoresis of the reduced protein with low iodine showed only rather small amounts of the two smaller sized (B and C) species and consisted of approximately 80% of the largest (A) species (Table II). A parallel experiment on the 0.68% iodine sample gave only about 10% of the largest species. It is evident that the trend observed between polypeptide chain composition and iodine content in the normal iodine guinea pig 19 S is preserved in the low iodine preparation.

Iodine Content of Reduced Chains of Thyroglobulin—Additional evidence for the relationship between iodine content and polypeptide composition of thyroglobulin was found by the determination of the iodine content of the isolated, reduced species separated by gel electrophoresis in Na dodecyl-SO₄ (Table III). It is clear that the iodine content of the three species isolated on Na dodecyl-SO₄ gels by electrophoresis increased with decreasing size of the polypeptide chains.
FIG. 4. Electrophoretic analysis of the polypeptide chain composition of reduced 19 S iodoprotein of different iodine contents and of reduced 27 S. The protein preparations and experimental conditions were the same as in Fig. 2 except that the disulfide bonds of the proteins were completely reduced with 2-mercaptoethanol (see "Methods"). The bands indicated by the letters A, B, and C represent species of molecular weights 295,000, 210,000, and 110,000. It should be noted that there is almost no difference in the gel pattern between 19 S and 27 S of similar iodine contents (compare 19 S e to 27 S).

DISCUSSION

It is generally found that complete reduction of the disulfide bonds will give the size of the elementary polypeptide chains in a protein if analysis is performed in a strongly dissociating solvent, such as Na dodecyl-\(\text{SO}_4\) or 6 M Gdm-Cl (14, 15). With the standard procedures, however, it has not been possible to resolve the polypeptide chain composition of thyroglobulin. Molecular weights between 80,000 and 200,000 have been reported for reduced-alkylated preparations of 19 S thyroglobulin examined by sedimentation equilibrium in denaturing solvents (1-3). Electrophoresis in Na dodecyl-\(\text{SO}_4\) gels of such preparations of thyroglobulin reveals an even greater diversity, since many bands representing much smaller sized molecules are observed, as well as bands representing the species already observed by centrifugation techniques (4-6). The reasons for such extreme heterogeneity in molecular size are not clear, although proteolytic breakdown has been suspected to be responsible for it (5, 16, 17).

The extensive heterogeneity of thyroglobulin observed with most species in different laboratories has precluded any simple conclusion about the composition of its elementary polypeptide chains, although a complicated schema has been suggested (6). It has been recently reported from this laboratory (7) that guinea pig thyroglobulin appears to be an exception, since approximately 95% of its reduced chains are distributed among three high molecular weight species having molecular weights of 295, 210, and \(110 \times 10^3\).

Such heterogeneity might result from modifications occurring during or after the exocytosis of the newly formed molecules from the thyroid cells into the follicular lumen. Carbohydrate addition and iodination are chemical modifications known to occur after the synthesis of the polypeptide chains of thyroglobulin. The first of these two is unlikely to contribute very much to polypeptide size heterogeneity, whereas iodine can act as an oxidant as well as undergo substitution into tyrosyl residues. It is well known that thyroglobulin, even if isolated from a single thyroid gland, consists of a mixture of protein molecules having different iodine contents, varying in most species anywhere from 0.2 to 1.0% (18-20).

For this reason, it was decided to observe the effects of increasing iodine content on the molecular species present in guinea pig thyroglobulin. Purified preparations of 19 S were fractionated according to their iodine content by isopycnic centrifugation in RbCl solutions. The composition of both the noncovalently linked subunits and the reduced elementary polypeptide chains was evaluated by electrophoresis in Na dodecyl-\(\text{SO}_4\) gels. Essentially the only product formed by dissociation of unreduced thyroglobulin in Na dodecyl-\(\text{SO}_4\) gels. Essentially the only product formed by dissociation of unreduced thyroglobulin in Na dodecyl-\(\text{SO}_4\) gels. The well known 12 S subunit, having one-half the molecular weight of 19 S (21, 22). The extent of dissociation was found to

FIG. 5. The polypeptide chain composition of (disulfide) reduced 19 S iodoprotein as a function of iodine content. All samples were obtained from RbCl gradients. The electrophoretic analysis in Na dodecyl-\(\text{SO}_4\)-polyacrylamide gels is described in Fig. 4. The relative amounts of A (\(\text{●}\)), B (O), and C (■) species were obtained by densitometry at 550 nm of the Coomassie blue-stained gels. The satellite bands of A (one faster band), B (a triplet), and C (one slower band), were considered to be identical in size with that of the major A, B, and C bands and were included in their densities. The sum of A, B, and C was always greater than 95%, but all of the visible bands were included in the calculation of the percentage of the major bands. The bands included in the percentage of A, B, and C are shown in Fig. 4. The points in Fig. 5 at identical iodine levels were obtained on separate electrophoretic analysis of the same protein fraction.
the 12 S subunits of 19 S are linked partly by disulfide bonds was not unexpected, since it has been previously shown that the decrease linearly with increasing iodine content. This result of reduced thyroglobulin isolated from all other species, it is species (295,000). Because of the more extensive heterogeneity highest in the smallest (110,000) and lowest in the largest sized varies inversely with the molecular size of each chain, being pig thyroglobulin indicates a relationship between iodination in view of the great similarity in their amino acid composition (7). This could be accounted for by their formation from extensive gene duplication of a primitive thyroglobulin gene of much smaller molecular weight. Fragments of thyroglobulin of molecular size smaller than that of the C species have been isolated with amino acid compositions very similar to that of the 19 S species (5).

The dependence of subunit composition of thyroglobulin on the degree of iodination could also be post-translational in origin. Iodination is known to cross-link 12 S subunits by formation of a disulfide bond from —SH groups (8) and to be involved in the conversion of 19 S to 27 S (27). A reduction in polypeptide chain size, however, must occur by peptide cleavage, unless there are nonpeptide bonds present in the chains. There is evidence that iodine can cleave peptide bonds adjacent to tryptophanyl (28) or tyrosyl (29) residues, although these reactions have not been reported with proteins. The present results may be accounted for if 19 S thyroglobulin consists of three domains connected by tyrosyl or tryptophanyl peptides which are split by iodine.

### Table II

**Dependence of polypeptide composition of 19 S guinea pig thyroglobulin on iodine content for two fractions of low (0.04%) and high iodine (0.68%) contents**

<table>
<thead>
<tr>
<th>Iodine</th>
<th>Composition (%)</th>
<th>Native</th>
<th>Reduced*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19 S</td>
<td>12 S</td>
<td>A</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>10</td>
<td>90</td>
<td>81</td>
</tr>
<tr>
<td>0.68</td>
<td>75</td>
<td>25</td>
<td>11</td>
</tr>
</tbody>
</table>

* A, B, and C designate the polypeptide species described in Fig. 4.

### Table III

**Iodine contents of polypeptide species A, B, and C**

The species A, B, and C were isolated as described elsewhere (7) from guinea pigs treated with thiouracil for 21 days.

<table>
<thead>
<tr>
<th>Species</th>
<th>Iodine content</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (M, 295,000)</td>
<td>0.01</td>
</tr>
<tr>
<td>B (M, 210,000)</td>
<td>0.12</td>
</tr>
<tr>
<td>C (M, 110,000)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

The iodine content in the isolated reduced chains of guinea pig thyroglobulin indicates a relationship between iodination level and the size of the polypeptide chains. The iodine content varies inversely with the molecular size of each chain, being highest in the smallest (110,000) and lowest in the largest sized species (295,000). Because of the more extensive heterogeneity of reduced thyroglobulin isolated from all other species, it is difficult, at present, to ascertain whether the above mentioned relationship holds true for other species or only for guinea pig.

The results indicate that any assessment of the subunit and polypeptide chain structure of thyroglobulin must include an analysis of the effects of iodination.

The effects of iodination could occur at the transcriptional, translational, or post-translational level. In the first case, some product(s) of iodination could regulate the expression of three separate genes, coding for the A, B, or C species; alternatively, the regulation of the synthesis of the three chains could be at the translational level as in the case of the α and β chains of hemoglobin by hemin (25, 26). Neither of these two possibilities can be excluded at the present time, although there is no direct evidence for either one. It does appear likely, however, that extensive homology exists among the A, B, and C chains in view of the great similarity in their amino acid composition (7).

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