Elementary Chain Composition of Guinea Pig Thyroglobulin

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Thyroglobulin obtained from guinea pigs was examined by Na dodecyl-SO₄-polyacrylamide gel electrophoresis after reduction and alkylation. In contrast to thyroglobulin from other mammalian sources, only three groups of polypeptide chains accounted for 95% or more of the protein. Determinations of the molecular weights of these purified proteins by equilibrium centrifugation in 6 M guanidine HCl gave values of 295,000 (species A), 210,000 (species B), and 110,000 (species C). Molecular weights determined by gel filtration in 6 M guanidine HCl gave similar results. Due to the large size of the polypeptides, satisfactory molecular weights could not be obtained from Na dodecyl-SO₄-polyacrylamide gel electrophoresis. Amino acid analysis of the three species was similar to that of whole thyroglobulin. Only slightly higher level of lysine and histidine and a lower level of glutamic acid were seen in species C. The iodine contents were found to range from 0.07 to 0.12 to 0.20% for species A, B, and C, respectively.

The noncovalent subunit structure of 19 S thyroglobulin is well known and consists of two similar or identical species with sedimentation constants near 12 S (1). Dissociation is favored by low ionic strength, alkali pH, low temperatures, and by a low iodine content of thyroglobulin (2, 3). More complete dissociation is produced by various denaturants, such as urea, Gdm-Cl, detergents, and organic solvents (4-6).

In contrast to our understanding of the subunit structure, we still lack precise information on the number and size of the polypeptide chains that form the 12 S subunit. These are held together covalently by approximately 60 disulfide bonds per subunit. Molecular weights of the reduced and alkylated polypeptide chains of 19 S thyroglobulin, determined by hydrodynamic methods in dissociating solvents, have ranged from 200,000 to 80,000 (7, 12). When the reduced alkylated protein is analyzed by electrophoresis in sodium dodecyl sulfates gels, as many as a dozen distinct bands can be seen (7, 8, 12). There are several possible explanations for the large number of apparent chains: (a) the electrophoretic pattern indicates the heterogeneity of the chains; (b) some of the larger species observed by electrophoresis represent aggregates of smaller ones; and (c) proteolytic activity is responsible for some of the smaller molecules. Prolonged incubation of thyroglobulin has been reported to produce smaller species (15).

A much simpler electrophoretic pattern has been found for homogeneous preparations of 19 S guinea pig thyroglobulin, where most of the smaller chains are absent. Three high molecular weight species account for approximately 95% of the total protein. The present work describes the isolation and characterization of these three species.

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§ The abbreviation used is: Gdm-Cl, guanidinium chloride.

METHODS

Preparation of 19 S Thyroglobulin and 27 S Iodoprotein—Thyroglobulin (19 S) and 27 S iodoprotein were isolated from male guinea pigs, National Institutes of Health inbred strain, as previously described (7). Extraction with 0.1 M sodium phosphate buffer, pH 7.2, in 0.02% sodium azide was followed by centrifugation at 30,000 x g, ammonium sulfate precipitation (1.3 M to 1.6 M), and gel filtration on Bio-Gel A-5m (Fig. 1). Ultracentrifugally homogeneous preparations of 19 S and 27 S were obtained from the Bio-Gel column. All steps were done at 4°. Protein concentration was measured spectrophotometrically (E TInt - 10.0).

Reduction and Alkylation—Reduction of the disulfide bonds was carried out at room temperature for 4 hours at a protein concentration of 1% in 6 M Gdm-Cl, 0.5 M Tris, and 0.002 M EDTA, pH 8.5, with a 100-fold molar excess of dithiothreitol per protein disulfide bond. The reduced proteins were alkylated at room temperature for 40 to 60 min with a molar ratio of iodoacetamide to dithiothreitol of 2.50:1. The pH was maintained at 8.5 by addition of 10 N sodium hydroxide. An excess of dithiothreitol was added to stop the reaction. The protein was dialyzed extensively against 6 M Gdm-Cl/0.05 M sodium phosphate, pH 7.15, when experiments were performed in Gdm-Cl solutions. When Na dodecyl-SO₄ was to be used as the dispersing agent, protein in the Gdm-Cl was first dialyzed against 6 M urea/0.05 M sodium phosphate, pH 7.2, and then against 0.06 M sodium phosphate/0.1% Na dodecyl-SO₄, pH 7.2. Determination of the number of alkylated cysteine residues in fully reduced and alkylated thyroglobulin gave the expected 240 residues of carboxymethylcysteine. It is difficult to be certain whether a few disulfide bonds may have escaped reduction. However, the similarity of the results obtained using different methods and conditions for reduction and isolation, i.e. different dissociation agents, temperatures, ratios of reactants, alkylating agents, times of reaction, and repetition of the entire procedure on the previously reduced and alkylated protein, indicates that reduction must be complete.

In some experiments thyroglobulin was reduced with 5% 2-mercaptoethanol in 0.05 M sodium phosphate/0.1% Na dodecyl-SO₄, pH 7.15, for 4 hours at 25°. A few electrophoretic experiments comparing this procedure with reduction and alkylation in 6 M Gdm-Cl (see above) were carried out to evaluate whether the two procedures gave the same results. Densitometry of Coomassie blue-stained gels showed no difference in the distribution of protein bands. Therefore, it was assumed that reduction with 2-mercaptoethanol in Na dodecyl-SO₄ was as efficient as reduction followed by alkylation in concentrated Gdm-Cl solution.

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Polyacrylamide Gel Electrophoresis—Electrophoresis in polyacrylamide gels was carried out in sodium dodecyl sulfate based on the method of Shapiro et al. (14). The total acrylamide concentration was normally 4% and the concentration of the cross-linking agent, methylenebisacrylamide, was 4% of the acrylamide. The samples were dissolved in 0.05 M sodium phosphate, 0.1% Na dodecyl-SO₄, and 0.02% sodium azide, pH 7.15, and electrophoresis was carried out in 0.1 M sodium phosphate/0.1% Na dodecyl-SO₄, pH 7.15, at a constant current of 8 mA/tube for 210 min. The gels were stained overnight with 0.1 M sodium phosphate/0.1% Na dodecyl-SO₄, pH 7.15, at a constant current of 8 mA/tube for 910 min. The gels were stained overnight in 0.02% Coomassie brilliant blue R-250 (w/v), 50% methanol (v/v), and 8.4% acetic acid (v/v) and were destained in 5% methanol/7% acetic acid (v/v) and 0.1 M sodium phosphate/0.1% Na dodecyl-SO₄, pH 7.15, to a constant absorbance of 0.600 to 0.650 at 595 nm.

Analytical Ultracentrifugation—Molecular weights were determined by sedimentation equilibrium using a split-beam photoelectric scanning system set at 280 nm. An AnG-Ti rotor and double-sector, Epon-filled 12-mm centerpieces were used. Equilibrium was ascertained by the constancy of the protein distribution during 12 hours. The determinations were carried out at 22°C on protein solutions of 0.15, 0.20, and 0.25 mg/ml in 6 M Gdm Cl/0.05 M sodium phosphate, pH 7.15. Speeds of 11,900 rpm were used for 12 S and species A and B, and 19,900 rpm for species C. Density values of 6 M Gdm Cl were obtained from the table of Kawahara and Tanford (16). Weight average molecular weight was calculated from the slopes of the plots of the logarithms of the concentration versus the square of the radial distance from the center of rotation. Values of 0.717, 0.7185, and 0.720 were used for the partial specific volumes of the A, B, and C species since the iodine contents were 0.07, 0.12, and 0.20%, respectively, for the three chains (17).

Amino Acid Analysis—Reduced and alkylated thyroglobulin fractions were hydrolyzed in 6 N HCl for 24 hours at 105°C in sealed and evacuated glass tubes. The amino acids were determined with a Beckman amino acid analyzer, model 120-C. All values are expressed as numbers of residues per 1000 residues of amino acid analyzed. No corrections were made for destruction of amino acids.

Iodine Analysis—Iodine analyses were performed by the Boston Medical Laboratories, using a modified Zak procedure (18).

**RESULTS**

*Native Thyroglobulin*—When thyroglobulins from seven species (guinea pig, hog, and rat) were analyzed by electrophoresis in Na dodecyl-SO₄ gels, two principal bands were always present (Fig. 2). We have shown that the faster migrating band has the same molecular weight as 12 S (see below). The slower migrating band must therefore be 19 S.

*Reduced Thyroglobulin*—When reduced and alkylated thyroglobulin was analyzed by electrophoresis in Na dodecyl-SO₄ gels, guinea pig thyroglobulin showed a different pattern from the other species. Fewer fast migrating bands were found and the pattern of the slower migrating bands was better resolved (Fig. 3). Three major molecular species (A, B, and C) were consistently seen in all guinea pig preparations and represented approximately 95% of the stained protein. The slowest moving species (A) migrates slightly slower than unreduced 12 S and was always present as a doublet with a minor and component. The A species appears to be the reduced form of 12 S since it has a similar molecular weight (see below). The intermediate species (B) was generally present as a doublet, although occasionally only a single band was seen. The fastest moving species (C) was always present as a major band with two satellite bands which represented about one-third of the C protein.

Gel chromatography in 0.1% Na dodecyl-SO₄ was undertaken to isolate the reduced and alkylated chains of guinea pig thyroglobulin since they were not soluble in water. A Bio-Gel A-15m column (1.6 x 90.5 cm), equilibrated with 0.05 M sodium phosphate, 0.1% of Na dodecyl-SO₄, and 0.02% sodium azide, pH 7.15, gave the elution pattern shown in Fig. 4. Every third or fourth tube was analyzed by electrophoresis in Na dodecyl-SO₄ gels and some of these are shown in Fig. 5. When a larger column (2.5 x 140 cm) was used for preparative purposes, identical results were obtained.

It can be seen from Fig. 5 that tube 95 contains almost pure species A. Species B is largely concentrated in tubes 101 to 100 with tube 105 containing more than 90% of B and only small amounts of A and C. The composition of the subsequent tubes changes rapidly and tube 113 shows approximately 90% of C. Chains smaller than C become visible in the electrophoretic patterns of the contents of tubes 119 and 123. The composition of the absorbance profile was calculated from the gel patterns and the relative amount of protein in chains smaller than C, estimated from the absorbance profile of the column, was consistent with the estimate from the Na dodecyl SO₄ gel experiments.

In order to isolate more homogeneous preparations of A, B, and C, tubes enriched in these species were pooled and rerun on the same Na dodecyl-SO₄ column. The eluates from the column showed no additional bands by electrophoresis in...
FIG. 2. (left). Polyacrylamide Na dodecyl-\(\text{SO}_4\)gel electrophoresis of guinea pig 19 S and 27 S, rat and hog 19 S, 1 to 2 mg/ml, 20 \(\mu\)l/tube (0.6 \(\times\) 8 cm), acrylamide concentration 4%. The proteins were dissolved in 0.1% Na dodecyl-\(\text{SO}_4\)/0.5 M sodium phosphate buffer. Electrophoresis was carried out in 0.1% SDS/0.1 M sodium phosphate, pH 7.15, at 8 ma/tube for 3½ hours. The gels are stained with 0.02% Coomassie blue.

FIG. 3. (right). Polyacrylamide Na dodecyl-\(\text{SO}_4\)gel electrophoresis of reduced and alkylated guinea pig 19 S and 27 S, rat and hog 19 S, 1 to 2 mg/ml, 25 to 40 \(\mu\)l/tube, acrylamide concentration 4%. All other conditions are given in Fig. 2. In the figure are indicated the guinea pig protein species A, B, and C.

Na dodecyl-\(\text{SO}_4\) gels, indicating that neither proteolysis nor dissociation had occurred during these procedures. Samples containing more than 90% of the principal components were obtained from the column eluates by selecting the tubes from the center portion of their absorbance peaks. The electrophoretic patterns of these purified samples are shown in Fig. 6.

When reduced and alkylated thyroglobulin was eluted from a Bio-Gel A-15m column equilibrated with 6 M Gdm-Cl/0.05 M sodium phosphate, at pH 7.2, the absorbance profile was very similar to that found on the Na dodecyl-\(\text{SO}_4\) column. Electrophoresis in Na dodecyl-\(\text{SO}_4\) gels of the polypeptides obtained from several elution tubes, after equilibration with the Na dodecyl-\(\text{SO}_4\) buffer system, gave the same results as was found with the protein from the tubes at the same relative position in the Na dodecyl-\(\text{SO}_4\) columns.

Properties of Polypeptide Chains A, B, and C—The Bio-Gel A-15m column, equilibrated with 6 M Gdm-Cl/0.05 M sodium phosphate, at pH 7.2, was calibrated with unreduced guinea pig thyroglobulin, phosphorylase b, and carbonic anhydrase. The elution volumes for the standards (expressed as \(K_d\)) (19) and for A, B, and C polypeptides from guinea pig thyroglobulin are shown in Fig. 7. The molecular weights obtained by column chromatography of the polypeptide chains in 6 M Gdm-Cl are listed in Table I. The molecular weights of the purified samples of A, B, and C obtained from the Bio-Gel A-15m column equilibrated with 6 M Gdm-Cl were determined directly by equilibrium centrifugation in the same solvent, i.e. 6 M Gdm-Cl/0.05 M sodium phosphate, pH 7.15. A linear dependence of \(\ln C\) with \(r^2\) was found for all samples. Three different polypeptide concentrations of each sample were evaluated. The differences in calculated weight average molecular weights were less than 5%. The values presented in Table I are mean values of the three determinations.
FIG. 5. Polyacrylamide Na dodecyl-So₄-gel electrophoresis of different fractions from the Bio-Gel A-15m column shown in Fig. 4. The sample concentration, gel concentration, and electrophoresis conditions were identical with the conditions given in Fig. 2.

FIG. 6. Polyacrylamide Na dodecyl-So₄-gel electrophoresis of species A, B, and C after final purification. All conditions for electrophoresis are identical with the ones given in Fig. 2.

Attempts were made to obtain the molecular weights of the A, B, and C species by electrophoresis in Na dodecyl-So₄ gels using well known proteins to calibrate the gels. The standard proteins (see "Methods") were first reduced with 5% 2-mercaptoethanol in 0.05 m sodium phosphate/0.1% Na dodecyl-So₄ solution, at pH 7.15, for 4 hours at 25°. Values obtained for the molecular weights of the A, B, and C species varied with the gel concentration in the range between 3 and 5.5%. It appears that this method of analysis is not satisfactory with the very high molecular weight molecules under consideration.

Amino Acids and Iodine Content—The amino acid composition of 19 S, A, B, and C, expressed as the number of residues per 1000 residues, is shown in Table II. No values for native guinea pig thyroglobulin have previously been reported. The amino acid composition of all the chains is similar to that of 19 S; the most variation is found in C where the proportion of lysine and histidine is slightly larger and that for glutamic acid is slightly smaller. The extensive agreement is more notable than the small differences in residue composition. Variations in the values for carboxymethylcysteine (results not shown) are probably due to oxidative reactions. Iodine analyses of species A, B, and C gave 0.07, 0.12, and 0.20%, respectively.

Properties of Reduced 12 S—Guinea pig thyroglobulin was dissociated into its 12 S subunits by incubation in 0.1 m sodium phosphate/0.1% Na dodecyl-So₄, pH 7.15, for 24 hours at 25°. The unreduced 12 S subunit of thyroglobulin was isolated on a Bio-Gel A-15m column equilibrated with the same buffer. Two peaks were obtained consisting of undissociated 19 S and the 12 S subunit.

The molecular weight of 12 S was measured by sedimentation equilibrium in the same 6 m Gdm-Cl solution used for the reduced polypeptide chains. A molecular weight of 330,000 was found from the slope of the plot of ln C against r². The electrophoretic mobility of 12 S was determined in the same Na dodecyl-So₄ gel used for the reduced polypeptide chains.
Fig. 7. Plot of $K_d$ versus log molecular weights. The Bio-Gel A-15m, equilibrated with 0.05 M sodium phosphate/6 M Gdm-Cl, pH 7.2, was calibrated with 12 S guinea pig thyroglobulin (1), phosphorylase b (2), and carbonic anhydrase (3). The elution of the protein species A, B, and C is shown by arrows.

### TABLE I

<table>
<thead>
<tr>
<th>Component</th>
<th>12 S</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td>Gel filtration in 6 M Gdm-Cl</td>
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<tr>
<td>12 S</td>
<td>330,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>320,000</td>
<td>295,000</td>
<td></td>
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<tr>
<td>B</td>
<td>225,000</td>
<td>210,000</td>
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<tr>
<td>C</td>
<td>120,000</td>
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<td>110,000</td>
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<td>Sedimentation equilibrium in 6 M Gdm-Cl</td>
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The migration rate of unreduced 12 S was similar to but slightly faster than the reduced A species. A small retardation in mobility is expected when comparing a reduced polypeptide with its unreduced form. The agreement in molecular weights and the approximate agreement in mobilities between A and unreduced 12 S make it appear that the former is produced by reduction of the latter without a change in molecular size.

### DISCUSSION

The analysis of unreduced thyroglobulin from guinea pig gives results that are very similar to those obtained with thyroglobulin from other species (8, 12). In the absence of reduction, denaturing agents only dissociate 19 S into the half-sized 12 S subunit. Furthermore, even extreme denaturing conditions do not completely dissociate all of the 19 S molecules. The degree of dissociation decreases as the iodine content of thyroglobulin increases (6, 21, 22). Since high iodine thyroglobulin has been shown to contain fewer SH groups than low iodine thyroglobulin, the possibility of interchain disulfide bonds preventing dissociation has been suggested (11, 23).

Electrophoresis in Na dodecyl-So₄ gels reveals over a dozen bands for most species (7, 12), whereas only three major band areas, all containing very high molecular weight polypeptides, comprise approximately 95% of the total protein in reduced and alkylated guinea pig thyroglobulin. It has not been possible so far to separate these three major groups of bands into individual components. Hence, it is possible that in species C, for example, each of the bands has a different amino acid composition. It seems more probable, however, that the differences are small and represent post-translational events, such as the addition of carbohydrate or deamidation. Heterogeneity in thyroglobulin as a whole has been found with respect to sialic acid and such microheterogeneity is known for other proteins (24).

A major problem is whether endogenous proteases could cleave a few highly susceptible bonds and cause the artificial appearances of the smaller polypeptide chains seen with other species by electrophoresis in Na dodecyl-So₄ gels.
Ambesi-Impiombato and Pitt-Rivers (13) have presented evidence that there may be intrinsic protease activity in thyroglobulin. Lissitzky and Rolland have found that incubation of purified preparations of hog thyroglobulin below pH 7 resulted in the disappearance of the slowly migrating and formation of the faster migrating bands. The multiplicity of fragments with different apparent molecular weights as seen in Na dodecyl-2SO₄ gel analysis of the thyroglobulin from many other species is in accord with a residual protease activity in thyroglobulin preparations. We regard this occurrence as unlikely with the preparation from guinea pig since very rapid and careful separation of thyroglobulin (see "Results") gave an electrophoretic pattern on Na dodecyl-2SO₄ gels almost identical with that given by preparations which had been prepared by the usual procedures. Storage of reduced alkylated guinea pig thyroglobulin for 1 year in the freezer also had no effect on the composition as analyzed by electrophoresis on Na dodecyl-2SO₄ gels.

The molecular weights of proteins as large as A, B, and C are difficult to determine by electrophoresis in Na dodecyl-2SO₄ gels. Hence, it was important to determine these values by methods which are more satisfactory for very high molecular weight polypeptide chains. We have found consistent results for the molecular weights of the three chains isolated from guinea pig thyroglobulin by two independent methods, both using 6 M Gdm-Cl. The latter solvent has proved to be the most effective in analyzing linear polypeptide chains (25). It should be pointed out that one method, i.e. equilibrium centrifugation, is a thermodynamic method and no assumptions are required to obtain molecular weights. The second method, using gel filtration, requires standards (see Fig. 7).

The molecular weights by the two methods of the three species are within experimental errors (10%) of each other. It should be noted that the molecular weight of the A species is similar to that of the 12 S subunit and presumably represents the random coil form of 12 S. The molecular weights obtained for the B and C species correspond, to a first approximation, to 2/3 and 1/2 of that of the A species. Taken at face value, this could imply that the basic polypeptide chain is 110,000 and the native thyroglobulin molecule is a hexamer, held together in part by disulfide bonds and in part by noncovalent interactions. If this is the case, however, one must account for the continued presence of the dimer and trimer species in spite of the complete reduction of the chains and the use of strongly dissociating conditions. Evidence to be presented elsewhere indicates that the B and C species are derived from the splitting of the A species.

The amino acid analyses (Table II) show a remarkable similarity amongst the three chains. The differences seen, primarily in the number of lysyl, histidyl, and glutamyl residues, are at the borderline of significance. This suggests that A, B, and C are related proteins. The level of iodination could be either the cause or the result of the difference in size of the three species.

REFERENCES

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² Unpublished data.
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