Evidence That Thyroglobulin Contains Nonidentical Half-Molecule Subunits*

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Bovine thyroglobulin was extracted from unfrozen glands, purified by sucrose gradient centrifugation, and fractionated into a narrow range in iodine content by RbCl isopycnic centrifugation. The subunit composition of these preparations was studied by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The extent of dissociation of 19 S into 12 S half-molecules followed the known relationship with iodine, i.e. decreased dissociability of 19 S with increased iodine content. The undissociated 19 S band always consisted of three closely spaced, equidistant bands. Limited reduction of the disulfides resulted in the isolation of an \( M_r = 160,000 \) molecule (1). Reduction of all the disulfide bonds produced polypeptides which varied in molecular weight from 160,000 to 300,000 by sedimentation equilibrium (7-9), whereas analysis by SDS-polyacrylamide gel electrophoresis revealed the presence of numerous bands with apparent molecular weights between 300,000 and 14,000 (10-12).

Hydroytic cleavage of peptide bonds during the preparative procedures was suspected of producing some of the lower molecular weight species (10, 13, 14). Recent reports indicated that proteolysis can be minimized in pig, sheep, and bovine glands by omitting the standard step of freezing prior to extraction of the protein (15, 16). In this case only traces of bands migrating faster than the unreduced 12 S half-molecule were seen in SDS-polyacrylamide gels. With this preparation, free of smaller degraded species, a molecular weight of 310,000 has been found for reduced alkylated 19 S thyroglobulin prepared from sheep and pig glands (17). In guinea pig preparations which are normally devoid of degraded species, three molecular species were isolated after reduction of 19 S thyroglobulin (18). However, it was suggested that the two smaller species (i.e. 210,000 and 110,000), which contained more iodine, may be derived from the 300,000 species which contained the least iodine (19). It has also been proposed that the 19 S obtained from cultured porcine thyroid cells was composed of two half-molecule subunits after reduction of the disulfide bonds (17). Biochemical studies of thyroglobulin with a 33 S mRNA isolated from bovine thyroid glands and translated by Xenopus oocytes also showed the formation of a molecule with a molecular weight of about 300,000 (20).

The only recent study in which more than one reduced species was isolated is that of Haeberli et al. (18). We now report the isolation of homogeneous preparations of three reduced molecular species from normally iodinated bovine thyroglobulin which differ from those of Haeberli et al. (18).

MATERIALS AND METHODS

Chemicals - Acrylamide monomer was purchased from Merck and recrystallized from chloroform; N,N'-methylenebisacrylamide was from Eastman Kodak; dithiothreitol and 5,5'-dithiobis(2-nitrobenzoic acid) from Sigma; SDS from Serva; GdmCl and urea (ultrapure) from Schwarc/Mann; 5-dimethylaminonaphthalene-1-sulfonyl chloride from Pierce Chemical Co.; suprapure RbCl was obtained from Eastman Kodak; dithiobis(2-nitrobenzoic acid) from Sigma; SDS from Serva; GdmCl and urea (ultrapure) from Schwarc/Mann; 5-dimethylaminonaphthalene-1-sulfonyl chloride from Pierce Chemical Co.; suprapure RbCl was obtained from Eastman Kodak; dithiobis(2-nitrobenzoic acid) from Sigma; SDS from Serva; GdmCl and urea (ultrapure) from Schwarc/Mann; 5-dimethylaminonaphthalene-1-sulfonyl chloride from Pierce Chemical Co.

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The abbreviations used are: SDS, sodium dodecyl sulfate; GdmCl, guanidium chloride; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
propagated in tubes (1.3 x 9 cm) with constricted lower ends which were sealed off with collection bags. The polypeptides were then eluted electrophoretically in the same buffer system at a constant current of 5 mA/tube. Elution was considered completed when the fluorescence had accumulated in a small volume at the tip of the collection bags. Excess buffer was siphoned from the top and the small volume of concentrated protein was removed with a Pasteur pipette and dialyzed overnight against 0.05 M sodium phosphate, 0.1% SDS (pH 7.2). Electrophoretic homogeneity was subsequently checked by subjecting an aliquot of the eluted polypeptides to analytical electrophoresis.

For analytical ultracentrifugation the peptides were freed from SDS by first dialyzing against 0.05 M sodium phosphate buffer (pH 7.2) which contained 6 M urea and then by addition of an equal volume of a thick slurry of Dowex 1-X2 (21). The mixture was incubated for 15 min at room temperature and the Dowex removed by passing through a Pasteur pipette plugged with glass wool. Analytical Ultracentrifugation — Molecular weights were determined by sedimentation equilibrium studies (28) carried out at 20° in a Beckman model E analytical ultracentrifuge equipped with an automatic photoelectric scanner with a 10-inch recorder and electronic speed control.

Reduced alkylated thyroglobulin polypeptides were dialyzed extensively against 8 M urea in 0.05 M phosphate (pH 7.2) before equilibrium centrifugation. Z-average molecular weights were calculated according to Yphantis (28) and Roark and Yphantis (29). A value of 0.719 ml/g was used for the partial specific volume of thyroglobulin in 8 M urea (22). Densities of the urea solutions were obtained from the data of Kawahara and Tanford (30). Our procedures of molecular weight determination were checked by analyzing the dimer and trimer of bovine serum albumin by the same techniques as we used for thyroglobulin. The serum albumin polymers were also purified by the methods as described for the separation of the reduced chains of thyroglobulin. The in c versus r² plots obtained from equilibrium centrifugation in 8 M urea were linear and gave molecular weights of 129,500 and 261,000 respectively, using a partial specific volume of 0.722 ml/g (31).
bond in 0.05 M phosphate, pH 7.2. Under these conditions all the disulfide bonds of thyroglobulin are reduced (11, 17, 18). The —SH groups were alkylated with a molar excess of 2.5:1 of iodoacetic acid to dithiothreitol by incubating for 30 min while the pH was controlled at 7.2 by addition of base. The solution was then dialyzed against 8 M urea, and finally against 1% SDS, in 0.05 M phosphate, pH 7.2. Electrophoresis in SDS-polyacrylamide gels revealed three reduced species, which will be referred to as S (slowest), F (fastest), and A (fastest). The three species were purified by preparative gel electrophoresis (see "Materials and Methods"). Only the same three species were observed by preparative as by analytical gel electrophoresis. Each purified component migrated at the same rate as in the mixture indicating that no equilibrium exists between the three species. Fig. 1 shows that the electrophoretic bands of purified A and F were symmetrical while a small shoulder was present on the descending limb of S, indicating minor contamination by F.

The molecular weights of the three species were determined in 8 M urea by equilibrium centrifugation. The data from the linear ln c versus r² plots were analyzed for Mw and Mr, and are summarized in Table I. It is clear that all three species have very similar molecular weights which correspond to that of the unreduced 12 S subunit. A similar result has been reported by Liszticki et al. (17) for reduced porcine thyroglobulin when sedimentation equilibrium was performed in 6 M GdmCl, pH 7.2. The closeness between the weight and z-average molecular weights supports the homogeneity of the reduced polypeptides S, F, and A.

**Iodine Content of Reduced Polypeptides**—Several preparations of thyroglobulin, after fractionation into different iodine contents by RbCl isopycnic centrifugation, were reduced. After separation by SDS-polyacrylamide gel electrophoresis the iodine contents of S, F, and A were determined (Table II). It is evident that the iodine content of each species varies considerably. In any one sample, however, the mobility increased as the iodine content of the species increased, S < F < A. It is of interest that 19 S which remained after reduction contained less iodine than all three smaller species in three of the samples and in two of the three smaller species in the fourth sample (Table II).

**Rate of Reduction**—The rate of formation of A, F, and S from un reduced 19 S and 12 S was evaluated by SDS gel electrophoresis (Figs. 2 and 3). The extent of disulfide reduction was also followed (Fig. 3).

Fig. 2 shows the scans of the gel patterns before and after thyroglobulin (0.35% iodine) was reduced for different times with an 8 molar excess of mercaptoethanol (per potential —SH group) in 1.0% SDS (pH 7.2), 0.05 M sodium phosphate at 20°. Before reduction the 12 S and 19 S (three peaks) represented 60 and 40%, respectively, of the protein. The rates of disappearance of the unreduced species as well as the rates of formation of the reduced species and the relative mobilities of the latter are shown in Fig. 3.

Only the rates of formation of the A and F species are summarized in Table II. It is clear that all three species have very similar molecular weights which correspond to that of the unreduced 12 S subunit. A similar result has been reported by Liszticki et al. (17) for reduced porcine thyroglobulin when sedimentation equilibrium was performed in 6 M GdmCl, pH 7.2. The closeness between the weight and z-average molecular weights supports the homogeneity of the reduced polypeptides S, F, and A.

**Experimental conditions and molecular weights of purified reduced polypeptides from sedimentation equilibrium centrifugation in 8 M urea**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Equilibrium speed</th>
<th>Concentration</th>
<th>Mr*</th>
<th>Mr*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>15,000</td>
<td>0.35</td>
<td>337,700</td>
<td>342,000</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>334,400</td>
<td>343,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>336,000±</td>
<td>342,000±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>15,000</td>
<td>0.48</td>
<td>317,500</td>
<td>320,700</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>325,500</td>
<td>333,340</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.43</td>
<td>313,000</td>
<td>340,300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.38</td>
<td>310,000</td>
<td>326,900</td>
<td></td>
</tr>
<tr>
<td></td>
<td>314,000±</td>
<td>337,000±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>15,000</td>
<td>0.31</td>
<td>328,000</td>
<td>337,000</td>
</tr>
<tr>
<td></td>
<td>0.29</td>
<td>312,800</td>
<td>319,700</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>296,800</td>
<td>320,900</td>
<td></td>
</tr>
<tr>
<td></td>
<td>319,000±</td>
<td>322,000±</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Weight average values obtained from ln c versus r².
+ Z-average values obtained by treatment according to Roark and Yphantis (29).

**Average.**

**Table II**

<table>
<thead>
<tr>
<th>Iodine contents (g/100 g) of reduced polypeptides compared to that of the native protein</th>
<th>Reduced polypeptides†</th>
<th>Native 19 S§</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 S</td>
<td>S</td>
<td>F</td>
</tr>
<tr>
<td>0.27</td>
<td>0.22</td>
<td>0.37</td>
</tr>
<tr>
<td>0.33</td>
<td>0.64</td>
<td>1.34</td>
</tr>
<tr>
<td>0.21</td>
<td>0.59</td>
<td>1.11</td>
</tr>
<tr>
<td>0.41</td>
<td>0.57</td>
<td>0.70</td>
</tr>
</tbody>
</table>

† Thyroglobulin (5 mg/ml) was reduced for 6 min at 100° in 1% SDS, 0.05 M sodium phosphate buffer (pH 7.2) with 100 molar excess of mercaptoethanol. These conditions should reduce essentially all the disulfide groups since incubation for 2 min with a 60 molar excess of mercaptoethanol (other conditions the same) formed 176 —SH groups/mol of thyroglobulin. The reduced polypeptides were separated by SDS-polyacrylamide gel electrophoresis and the relative composition determined by densitometry of the Coomassie blue-stained gels. The individual polypeptide bands were sliced out and dissolved in H2O, and the iodine content was determined as described under "Materials and Methods." The per cent recovery ranged between 80 and 110% of the total amount of iodine applied on the gel.

§ Fractionated into different iodine contents by RbCl isopycnic centrifugation.
**Nonidentical Subunit Structure of Thyroglobulin**

**Fig. 2.** Scans of SDS-polyacrylamide gels of unreduced thyroglobulin (0.35% iodine) and the same preparation which was reduced in 0.05 M sodium phosphate (pH 7.2), 1.0% SDS with 8 molar excess of mercaptoethanol at 20° for the indicated periods of time. The reduction was terminated by addition of 2.5 molar excess iodoacetic acid per -SH group with control of the pH at 7.2 with 1 M NaOH. The horizontal lines indicate the decrease in mobility of the reduced components relative to the origins of the gels and the position of unreduced 12 S.

**Fig. 3.** Change in composition and mobilities of thyroglobulin polypeptides with increasing time of reduction. Experimental conditions are identical to those in Fig 2. -SH groups were determined with Ellman's reagent according to the procedure of de Crombrugghe et al. (24).

obtainable directly from the gel scans. The A and F species appear in the earliest gel scan (i.e. 2 min) and migrate at faster rates than unreduced 12 S (Figs. 2 and 3). It should be noted that the S species migrates at the same rate as 12 S and is never resolved from it. Therefore the electrophoretic peak labeled S represents the sum of the reduced S and unreduced 12 S species as long as the electrophoretic composition changes with time. Consequently only the rate of formation of F and A is unambiguous.

The rate of disulfide reduction is clearly slower than the rate of disappearance of 19 S or the rate of formation of F or A (Fig. 2). After 80 min of reduction, the amounts of A, F, and S did not change any more while less than half of the disulfide groups were reduced. Further reduction, however, does produce small changes in mobility of the three new species.

When the same experiment was repeated with a high iodine preparation of thyroglobulin (0.98%), the reaction proceeded at about the same rate (Fig. 4). After 80 min the relative amounts of F and S were about the same but twice as much A was present. In this preparation there was much more of unreduced 19 S than 12 S present. The amount of protein in the S peak did not change significantly with time since it is formed from unreduced 19 S at the same rate that 12 S disappears to make F and A.

The mobilities of the various species obtained from the low iodine preparation are seen in Fig. 3. There is a decrease in mobility of the S, F, and A species with increasing extent of reduction. The changes with the higher iodine preparation, although in the same direction, were smaller.

When different thyroglobulin preparations, containing between 0.2 and 1.0% iodine, were fully reduced the relative amounts of S and F varied between 40 and 60%. We have, however, obtained preliminary results which indicate that below 0.2% iodine there is a greater change in the relative amounts of S and F.

**DISCUSSION**

Only two bands are observed by electrophoresis in SDS gels with unreduced thyroglobulin, the 19 S and 12 S. The 19 S band contains three peaks, representing three closely related 19 S species.

The reduction of all the disulfide bonds of thyroglobulin results in the formation of two major and one minor, new electrophoretic species. The differences in mobility are due to differences in hydrodynamic volume, i.e. Stokes radius, since the molecular weights of the three species are indistinguishable, i.e. 330,000.

The Stokes radius, as determined by viscosity, gel filtration, 4 To be published elsewhere.
weight if the shape of the polypeptides being compared is the same, i.e., rigid rod-like or flexible random coils (33-37). For the mobility (in SDS gels) to be related to the Stokes radius, not only the shape but also the charge density should not vary. It has been assumed that a constant charge density occurs with reduced polypeptide chains since in two studies of SDS binding a constant amount of SDS was bound (1.4 g/g of protein) in excess SDS (34, 38). However, a much more detailed, recent study with a large number of proteins revealed a greater diversity in the binding isotherms and in the maximum amount of SDS bound (39). The differences in mobility can, therefore, be understood if the extent of SDS binding to the three reduced species of thyroglobulin is different.

It has also been reported that the maximum binding of SDS is greater for reduced than for unreduced forms of the same protein (34, 38). The decrease in mobility of A, F, and S with reduction can be explained by an increase in the Stokes radius since increased binding of SDS with reduction would increase the mobility in the absence of a shape change.

There is another conceivable explanation of the differences in mobility of S, F, and A if we make the assumption that there are cross-links which, at least in F and A, are not disulfide. If these are far enough apart along the chain they would decrease the hydrodynamic volume. There is no evidence for bonds of this type, but new types of bonds are certainly possible during the peroxidatic iodination of thyroglobulin in the gland to form the thyroid hormones, thyroxine, and triiodothyronine, and their precursors. Peroxidases are known to oxidize many compounds as well as cross-linking phenolic groups (40, 41).

It has been found that the mobility increases with the iodine content, i.e., A > F > S. It is highly unlikely that the differences in iodine content are responsible for those in mobility since the iodine content in S found in one fraction was greater than found in F in another fraction (Table II). Moreover, each fraction of thyroglobulin prepared by RbCl gradient centrifugation contains a distribution of iodinated molecules which overlap in iodine content (see Table II). If the electrophoretic separation between A, F, and S depended on iodine levels, one would not expect to obtain widely separated mobilities for the three reduced species.

The variations in mobility could also be accounted for if there were significant differences in carbohydrate composition in the three reduced species. If this were the case, it is difficult to understand why the unreduced species are not separable when examined under the same conditions as the reduced species. Moreover, analysis of the neutral sugars by the anthrone reaction indicated that there were no significant differences between S, F, and A. Treatment of thyroglobulin with sialidase also did not change the composition or mobilities of the three reduced species as compared to the untreated control.

Earlier data have reported the existence of two 12 S species in thyroglobulin. Velocity sedimentation of human thyroglobulin ( unreduced), in dilute alkalai and low salt, has resolved two closely sedimenting 12 S species (4). Electrophoresis of reduced alkylated bovine thyroglobulin in polyacrylamide gels containing 8 M urea at neutral pH also showed two clearly separated bands (3). It seems likely therefore that the subunits of thyroglobulin are comprised of two major polypeptide chains of similar molecular weight, but with slightly different amino acid composition. Preliminary amino acid analyses of S, F, and A showed a few differences between each other and with native 19 S. However, the individual polypeptide chains probably contain domains of homologous amino acid sequences since numerous amino acid analyses of proteolytic fragments of 19 S thyroglobulin, varying widely in size, have failed to reveal any gross differences in composition (10-12).

The present results are not incompatible with those of Rolland and Lissitzky (15) since, although they reported the isolation of one reduced polypeptide chain of molecular weight 310,000, Fig. 6 in their paper shows that in SDS-polyacrylamide gel electrophoresis the band for the reduced proteins is much broader than that for the unreduced or, more likely, there are two bands. Moreover in Fig. 8, the electrophoretic mobility of their fastest migrating band increases with increasing fraction number (i.e., 86 to 96). The different fractions were obtained by gel filtration of reduced thyroglobulin on Bio-Gel A-5m in 0.1% SDS, pH 7.2.

The subunit composition of guinea pig thyroglobulin reported by Haefeli et al. (15, 19) remains significantly different from that of porcine and ovine reported by Rolland and Lissitzky (15) and that presently reported. The reason for this difference is not apparent at present.

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Nonidentical Subunit Structure of Thyroglobulin

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