The Subunits of Thyroglobulin

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1. Pig thyroglobulin was reduced with dithiothreitol in the presence of sodium dodecyl sulphate, 8M-urea or 6M-guanidinium chloride. 2. The molecular weights of the reduction products were about 165 000. 3. Two stable subunits with molecular weights as low as 35 000 and 20 000 were separated by Sephadex chromatography after prolonged exposure of the reduction products to sodium dodecyl sulphate at pH 8.7. Although a hydrolytic reaction is probably implicated, the nature of the chemical linkages broken was not established.

In most mammalian species thyroglobulin is a heterogeneous protein whose principal (80%) component has a sedimentation coefficient of about 19S and a mol.wt. ~660 000. Two heavier components (27S and 31–34S) and three lighter components are also present (Shulman, Rose & Witebsky, 1955; Robbins, 1961; Salvatore, Salvatore, Cahnmann & Robbins, 1964). Heat, alkali or denaturing agents cause the dissociation of some 19S thyroglobulin into 12S subunits with mol.wt. ~330 000 (Edelhoc & Rall, 1964); in 9M-urea thyroglobulin exists in approximately equal amounts as the half molecule and a molecule with a mol.wt. only slightly lower than that of the native protein (Edelhoc & Steiner, 1966). When the thyroglobulin is deficient in iodine, dissociation of the 19S molecule occurs readily and it was suggested (de Crombrugge, Pitt-Rivers & Edelhoc, 1966; de Crombrugge, 1968; Ui & Tarutani, 1968; Schneider & Edelhoc, 1969) that 19S thyroglobulin exists in two forms: in the labile form the two 12S molecules are joined by non-covalent bonds, whereas in the stable 19S form the 12S subunits are joined by one or more disulphide bridges.

Thyroglobulin (19S) contains 202 half-cystine residues (Edelhoc & Rall, 1964), most of which are present as disulphides although thyroglobulin in several species can contain up to five thiol groups/molecule (Pitt-Rivers & Schwartz, 1967).

Pierce, Rawitch, Brown & Stanley (1965) described the reduction and carboxymethylation of human thyroglobulin and the determination of the mol.wt. (110 000–125 000) of the subunits in the presence of SDS.† In two experiments only they

obtained subunits with mol.wt. 50 000–60 000. de Crombrugge *et al.* (1966) obtained subunits with mol.wt. 185 000–230 000 by mercaptoethanol reduction and carboxymethylation of calf thyroglobulin in 8M-urea; no smaller reduction product was obtained and this subunit was called thyroglobulin monomer. They believed that 12S thyroglobulin had been dissociated into two identical or similar subunits of mol.wt. 165 000. In lower concentrations of urea the monomer aggregated to a dimer with a sedimentation coefficient only slightly lower than 12S. Lissitzky, Rolland, Reynaud, Savary & Lasry (1968) concluded from their experiments on reduced carboxymethylated sheep thyroglobulin in guanidinium chloride, SDS and urea that the 12S protein consists of two different types of subunit with identical mol.wts. ~80 000.

Preliminary electrophoresis of fully reduced alkylated thyroglobulin in polyacrylamide gels in 8M-urea indicated that a separation of two subunits might be possible (Edelhoc, 1965). The present work was undertaken to pursue these findings; however, our experiments on electrophoretic separation of reduced alkylated thyroglobulin subunits in polyacrylamide gels containing urea or SDS were totally unreproducible. In apparently similar conditions the gels showed 1–8 protein bands after staining with Amido Black; even in ultracentrifugally monodisperse preparations multiple bands were obtained. We therefore abandoned this method and tried separation by gel filtration.

MATERIALS AND METHODS

Dithiothreitol (Cleland's reagent) was obtained from Calbiochem Ltd., London W.1, U.K. SDS (specially pure), glycine, guanidinium chloride and iodoacetamide were from British Drug Houses Ltd., Poole, Dorset, U.K.; iodoacetamide was crystallized from ethanol. 2-Amino-2-methylpropan-1-ol was from Koch-Light

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† Abbreviation: SDS, sodium dodecyl sulphate.

Laboratories Ltd., Colnbrook, Bucks., U.K., tris from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and urea from May & Baker Ltd., Dagenham, Essex, U.K. Sephadex G-200 was from Pharmacia (G.B.) Ltd., London W.5, U.K. Pig thyroglobulin (19S) was prepared by Mr A. D. Brownstone (1969); the product was ultracentrifugally and electrophoretically monodisperse and was stored in 50 mg batches in tris-glycine-acetic acid buffer, pH 7.8, at -20°C to avoid repeated freezing and thawing of the solutions.

Reduction and alkylation. (a) Reduction in SDS. Reduction was carried out on 50–400 mg of 19S thyroglobulin; the protein solution, containing about 10 mg/ml, was held at pH 9 with 2-amino-2-methylpropan-1-ol and was treated at room temperature for 16 h with SDS (1.5 mg/ml) to unfold the molecule sufficiently for complete reduction. Dithiothreitol was then added in an amount equivalent to 100 mol/mol of disulphide in the protein; reduction and alkylation were carried out at pH 9 (approx.). After 4 h iodoacetamide (2 equiv./mol of thiol) was added with vigorous stirring and left to react for 1 h. In early experiments lower concentrations of dithiothreitol and iodoacetamide were used, but the amounts of both reagents were later increased to obviate the risk of reoxidation of the thiol groups. The reaction mixture was dialysed against repeated changes of buffer (pH 8.7) containing 0.05 M-glycine, 0.0165 M-tris, 0.1 M-NaCl, 0.02% NaN_3 and 0.03% SDS (R buffer). The SDS concentration was kept below the critical micelle concentration for 0.1 M-NaCl (Williams, Phillips & Mysels, 1955). Dialysis was continued for 8 days to obtain maximal binding of the SDS to the protein. The molecular weights found after reduction in SDS were lower than those found in urea and guanidinium chloride (see the Results and Discussion section); this implies that the subunits were further breaking down during the equilibration period. Reduction in SDS was therefore repeated as described above, but after removal of excess of iodoacetamide etc. by a 3 h dialysis against tris-glycine buffer, pH 8.7, 0.75 mg of SDS/mg of protein was immediately added and further dialysis against complete R buffer was carried out overnight. Ultracentrifugal analysis of the product was done within 48 h of the reduction. Bound SDS was estimated by the method of Karush & Sonenberg (1950) as modified by Pitt-Rivers & Ambesi Impiombato (1968). The protein concentration was estimated from the extinction of the solution at 280 nm (Edelhoc & Lippoldt, 1962).

(b) Reduction in guanidinium chloride. The thyroglobulin solution was dialysed to equilibrium against 6 M-guanidinium chloride, pH 8.7, and reduced for 30 min as above; after alkylation for 30 min it was further dialysed for several days against 6 M-guanidinium chloride, pH 8.7, containing 0.1 M-NaCl.

(c) Reduction in urea. The thyroglobulin solution was dialysed to equilibrium against neutral 8 M-urea and held at pH 10 with 2-amino-2-methylpropan-1-ol. After reduction for 30 min and alkylation for 30 min the reaction mixture was dialysed against repeated changes of 8 M-urea, pH 8.7, containing 0.1 M-NaCl.

Gel filtration. Sephadex G-200 was equilibrated with R buffer for 2 days and packed into columns 1.4 cm $\times$ 100 cm for loads of 25 mg or less of protein and 2.0 cm $\times$ 120 cm for loads up to 80 mg of protein. Peaks were monitored in an LKB Uvicord 2 at 280 nm as they passed

to a fraction collector. Appropriate fractions were precipitated by dialysis against repeated changes of aqueous acetic acid, pH 3.5, containing 0.2 M-NaCl and 0.02% NaN_3 , until the extinction of the supernatant at 280 nm showed that precipitation was complete. After they had been centrifuged the precipitates were redissolved and dialysed against the required buffer (SDS, urea or guanidinium chloride) for ultracentrifugal analysis. Dialysis was at room temperature with continuous stirring.

Sedimentation-velocity experiments. These were done at 59780 rev./min and approx. 20°C (or 25°C for samples in urea or guanidinium chloride) in 12 mm ordinary or synthetic-boundary cells in a Spinco model E ultracentrifuge equipped with a temperature-control unit. The records obtained with the schlieren phase-plate optics (λ 546 nm) were measured and sedimentation coefficients, corrected to water at 20°C , expressed in Svedberg units (S). For this we measured the relative viscosity of the solutions with standard U-tube viscometers. It also required some assumptions about $\bar{v}$, the partial specific volume (see below).

Molecular-weight measurements. These were all done at 25°C in a short-column multi-channel cell (Yphantis, 1960) as described by Charlwood & Utsumi (1969). Final patterns were recorded after 3–3.5 h. Concentrations of stock solutions were measured refractometrically (Cecil & Ogston, 1951) or, for samples in urea or guanidinium chloride, from peak areas in synthetic-boundary cells. Dilutions were done with accurate microsyringes (Scientific Products Inc., Flushing, N.Y. U.S.A.). For buoyancy factors the data of Kawahara & Tanford (1966) were used for urea and guanidinium chloride, and the partial specific volume of the protein was taken as 0.723 (Derrien, Michel, Pedersen & Roche, 1949). However, where SDS was bound to the protein a corrected $\bar{v}$ was calculated assuming volume additivity and a specific volume of 0.885 for SDS, and the weight of bound SDS was subtracted from the overall molecular weight (Criddle, Bock, Green & Tisdale, 1962).

RESULTS AND DISCUSSION

Ultracentrifugal analysis of the products of six reductions in SDS with prolonged exposure to SDS at pH 8.7 gave molecular weights ranging from 55 000 to 66 000. These results agree with two isolated findings of Pierce *et al.* (1965) where molecular weights of 50 000 to 60 000 were recorded; these values were only about half the value found by these authors in all other cases.

Fig. 1 shows molecular weights of the products of reduction of thyroglobulin in SDS without prolonged equilibration at pH 8.7, and of two products obtained by reduction and alkylation in 8 M-urea and 6 M-guanidinium chloride respectively. Although it is impossible to extrapolate to zero concentration very accurately, the values for the molecular weight of fully reduced 19S thyroglobulin thus obtained are in the same range as those recorded by de Crombrughe *et al.* (1966) and suggest that reduction alone does not give a molecule smaller than thyroglobulin monomer.

Fig. 1. Apparent molecular weights of products obtained by reduction of thyroglobulin in the presence of SDS (●), 6M-guanidinium chloride (■) and 8M-urea (▲). In all cases in addition to these components the solvent included 0.1M-NaCl and 50mM-glycine-tris buffer, pH 8.7.

It appears that, even under quite mild conditions, reduction of thyroglobulin in SDS gives further breakdown products. Exposure to slightly alkaline conditions (pH 8.7) at room temperature for 8 days or storage of reduced alkylated thyroglobulin at 4°C or in the frozen dried state in the presence of SDS could account for the lower molecular weights (compared with those obtained in urea or guanidinium chloride) found by us and by Pierce *et al.* (1965). It is noteworthy that prolonged storage of reduced alkylated thyroglobulin in urea at pH 8.7 (2 years at -20°C) did not cause extensive breakdown of the monomer.

Spontaneous breakdown of thyroglobulin monomer in SDS. Pierce *et al.* (1965) observed that solutions of reduced alkylated thyroglobulin stored at -20°C for 3 months deteriorated and no longer showed a single peak in the ultracentrifuge, but no details of the breakdown products were given. Two of our reduced alkylated thyroglobulin preparations in SDS, stored at room temperature for 12 weeks and at -20°C for 12 months, both at pH 8.7, were analysed on Sephadex G-200 and gave two symmetrical retarded peaks equivalent to about 80% of the total material. The faster product, B (about 30%), and the slower product, C (about 50%), were freed from SDS by acid precipitation and equilibrated with 6M-guanidinium chloride, pH 8.7, containing 0.1M-sodium chloride. In this solvent they had molecular weights of about 35 000 and 20 000 respectively (Fig. 2). Subsequent analyses of other reduction products stored in SDS in the cold or at 37°C failed to reveal any products other than B and C, which always appear in approximately the same relative proportions. B and C are relatively stable and are not broken down in SDS at pH 8.7 even when the solutions are heated at 85°C for 20h.

Fig. 2. Molecular weights of column fractions R111B (●) and R111C (■) measured in 6M-guanidinium chloride, 0.1M-NaCl and 50mM-glycine-tris buffer, pH 8.7. Regression lines are shown but they are of doubtful significance because of the scatter.

The nature of the cleavage of thyroglobulin monomer to products of lower molecular weight (120 000 to 50 000) is not known but hydrolysis seems the most likely explanation; further hydrolytic cleavage could also account for the formation of the products B and C and this hydrolysis seems to be accelerated by SDS. The optimum concentration of SDS and the composition of B and C are being further investigated.

Heterogeneity of thyroglobulin monomer. One factor strongly indicates that thyroglobulin monomer obtained by reduction of monodisperse 19S protein is not homogeneous: on the Sephadex G-200 column the subunits obtained by reduction in guanidinium chloride, urea or SDS always show an unsymmetrical peak that emerges at the front of the column and frequently splits into two. Occasionally two shoulders appear on the trailing edge of this peak which indicate the presence of at least three components in this fraction. This could be accounted for if there were more than one species of 12S protein. This possibility was discussed by Edelhoch (1965) but has not been experimentally confirmed.

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