THE CONSTITUENT POLYPEPTIDE CHAIN OF PORCINE THYROGLOBULIN

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1. Introduction

Thyroglobulin the major iodinated protein of the thyroid gland has a mol. wt. of about 660 000 for a sedimentation coefficient of 19 S [1]. In conditions which do not break covalent bonds, it dissociates more or less completely into half-sized 12 S subunits.

In a previous paper [2] we have shown that ultracentrifugally homogeneous porcine and ovine 19 S thyroglobulin were contaminated with acidic and/or alkaline proteolytic activity. Thyroglobulins prepared from fresh glands at pH 7.2 do not self-digest at acidic pH but still contain an endogenous protease activity with a pH optimum at 8.6. Inhibition of the latter activity by diisopropylfluorophosphate, 6 M guanidinium·HCl and 8 M urea was demonstrated. In conditions where the action of endogenous protease activity is strongly inhibited, polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS) of the fully reduced and *S*-alkylated protein showed a major protein band migrating at the same rate as the unreduced 12 S subunit. No faster moving bands were observed suggesting an apparent mol. wt. identical to that of the native 12 S for the constituent peptide chains of hog and sheep thyroglobulins.

In the present letter, this was confirmed by sedimentation equilibrium studies and analyses by SDS-polyacrylamide gel electrophoresis of poorly-iodinated and iodine-rich ^{125}I-labeled thyroglobulins obtained from thyroid cells reassociated into follicles in culture conditions.

2. Materials and methods

Thyroglobulin was purified from fresh porcine glands by slice extraction, precipitation with buffered ammonium sulfate and Sepharose 6-B gel filtration as previously described [2]. All buffers were at pH 7.2 and contained 0.02% sodium azide. All steps were performed at 0–2°C.

Isolated thyroid cells were obtained by trypsinization of porcine thyroid glands [3]. Freshly dispersed cells were suspended at a concentration of 3×10^6 cells/ml in Eagle medium containing 10% calf serum, streptomycin sulfate (50 μg/ml) and penicillin (200 U/ml). Falcon plastic Petri dishes (3.5 cm diameter) were seeded with 1.5 ml cell suspension supplemented with 4 mU/ml porcine thyrotropin and incubated for 2 days at 35°C in 95% air–5% CO_2. The medium was then discarded and replaced by the same fresh medium enriched with iodide-free calf serum obtained by extensive dialysis. In a first series of experiments, the medium of each dish for a constant vol of 1.5 ml was supplemented with 0.1 ml carrier-free ^{125}I as NaI at time zero of postincubation and 12, 24 and 36 h after. In the second series, radioactive iodide was enriched with $Na^{127}I$ to obtain at each addition step a theoretical final concentration of 1 μM. After 48 h incubation at 35°C in 95%–5% CO_2, the final concentration of stable iodide measured in the culture medium was 1.5 μM.

After 48 h incubation, the cell layer was washed twice with 2 ml cold PBS containing in g/litre: NaCl 8, KCl 0.2, Na_2HPO_4 2.89, KH_2PO_4 0.2, $CaCl_2$ 0.066, $MgCl_2$ 0.1 and glucose 1. The follicles were recovered by gentle scraping in 1 ml PBS and homogenized by hand with 4 strokes of a very loosely-fitting Dounce homogenizer. The homogenate was centrifuged at 10 000 *g* for 10 min and the supernatant was filtered on a Sephadex G-25 column (1 × 25 cm) equilibrated

with 0.05 M sodium phosphate pH 7.2, 0.15 M NaCl to separate protein-bound iodine from iodide. The excluded peak fractions were pooled and filtered at a flow rate of 4.5 ml/h on a Sepharose 6-B column (1.3 × 80 cm) equilibrated with the same buffer. A single peak of radioactivity eluted in the same volume as marker 19 S thyroglobulin. All operations of cell homogenization and thyroglobulin purification were done at 0–2°C.

In other experiments, 10 ml of fresh thyroid cell suspension were seeded in 10 ml plastic Petri dishes 'not for tissue culture'. The medium was supplemented with 0.25% gelatin instead of thyrotropin [4]. In the same conditions of incubation, these cells form three-dimensional follicles *in suspension.* After 5 days incubation, the follicles were recovered by centrifugation (5 min at 100 *g*) and washed once with PBS. The cells were suspended in 2 ml PBS containing 0.5 μM iodide and incubated in stoppered tubes for 1 h at 35°C in the presence of 2 μCi ^{125}I. After washing by sedimentation, the cells were homogenized and ^{125}I-thyroglobulin was purified as described above.

Reduction of purified gland thyroglobulin was performed as previously described [5] for 4 h at room temperature in 6 M guanidinium·HCl, 0.1 M sodium phosphate pH 7.2 with a molar excess of 100 mol of dithiothreitol per protein disulfide bond. Alkylation of thiol groups formed was carried out with iodoacetamide (2.24 mol/mol dithiothreitol). The modified protein was then extensively dialyzed against 6 M guanidinium·HCl, 0.1 M sodium phosphate pH 7.2. Amino acid analysis of the reduced protein showed the absence of cystine and the expected number of carboxymethylcysteine.

Electrophoresis was performed in 0.5 × 10 cm polyacrylamide gels (4% total acrylamide concentration and 4% methylene bisacrylamide (MBA) to acrylamide) in 0.05 M Tris-glycine pH 8.4 containing 0.1% SDS as first described by Shapiro et al. [6]. Gels were stained overnight with 0.02% Coomassie brilliant blue and destained as indicated by Fairbanks et al. [7]. Electrophoresis without SDS was performed according to Ornstein [8] using 5% total acrylamide concentration and 2.4% MBA in 0.36 M Tris–Cl pH 8.9 for the running gel, and 4% total acrylamide concentration and 25% MBA in 0.06 M Tris-Cl pH 6.7 for the stacking gel. Distribution of radioactivity in gels was determined on about 2 mm width sections and counting in a Packard Autogamma spectrometer.

Sedimentation velocity experiments were performed at 20°C in a Spinco model E ultracentrifuge equipped with interferometric and Schlieren optics. Mol. wts. were determined by sedimentation equilibrium of reduced and S-alkylated thyroglobulin in 6 M guanidinium·HCl, 0.1 M sodium phosphate pH 7.2 using the meniscus depletion method. The AnD rotor and double sector, epon filled 12 mm center pieces were used. The speed was 10 000 rev/min, the temperature 20°C and the protein concentration, 0.4 mg/ml. Equilibrium was ascertained by the constancy of protein distribution for 36 h. Weight average mol. wts. were computed from the slopes of plots of log C vs r^2. The value of 0.719 was used for the partial specific volume. Density values of 6 M guanidinium·HCl were obtained from the table of Kawahara and Tanford [9].

3. Results and discussion

Ultracentrifugally homogenous 19 S thyroglobulin purified from porcine glands and analyzed by electrophoresis in SDS-gels showed a fast moving band migrating at the same rate as the native purified 12 S subunit and 3 slower migrating bands (fig.1). Thus the latter must be 19 S thyroglobulin. Why its resolves into 3 discrete components is still unknown. Previous hydrodynamic studies [10] demonstrated that the 12 S porcine subunit has a mol. wt. of about 330 000 i.e. half the size of 19 S.

After reduction and *S*-alkylation, porcine 19 S thyroglobulin sedimented as a single homogenous boundary in 6 M guanidinium·HCl, 0.1 M sodium phosphate pH 7.2 (not illustrated). The mol. wt. of the reduced and alkylated protein was determined by sedimentation equilibrium in this solvent. A linear dependence of log C vs r^2 was found (fig.2). In three different thyroglobulin preparations average molecular weights of 315 000, 310 000 and 310 000 were obtained.

Thyrotropin-stimulated thyroid cells reassociate into follicles in culture and accumulate part of the synthesized thyroglobulin in the follicular lumen [11,12]. As shown by light microscopy mild conditions of homogenization (see Materials and methods) disrupt the follicular structure and permit the release of col-

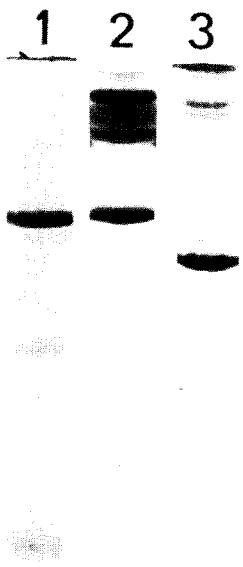

Fig.1. Polyacrylamide SDS gel electrophoresis of porcine gland thyroglobulin (Gel 2). Gel 1, purified 12 S thyroglobulin subunit obtained from porcine thyroid cell monolayer cultured in the absence of iodide; gel 3, myosin. Electrophoresis was performed in 0.1% SDS, 0.05 M Tris-glycine pH 8.4 at 4 mA/tube for 45 min. Gels stained with Coomassie blue.

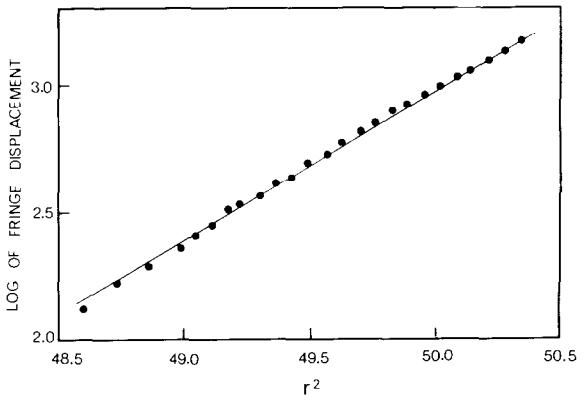

Fig.2. Sedimentation equilibrium of reduced and *S*-alkylated porcine thyroglobulin in 6 M guanidinium-HCl, 0.1 M sodium phosphate pH 7.2.

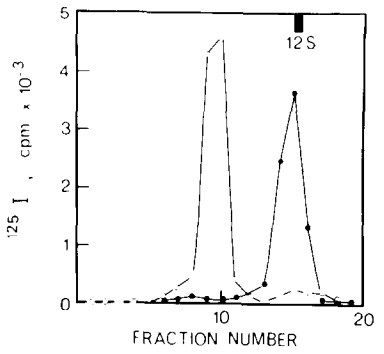

Fig.3. Electrophoresis in polyacrylamide gels of poorly-iodinated ^{125}I-thyroglobulin purified from cultured follicles in the absence of stable iodide in the culture medium. (○) Untreated protein; (●) after treatment with 1.5 mM SDS for 1 h at room temperature before electrophoresis. Migration was for 2.5 h at 2.5 mA/tube. Gels were cut into 34–35 sections; no radioactivity was found in sections 20 to 34–35. The black bar represents the position of migration of native 12 S subunit as shown by Coomassie blue staining. Distribution of radioactivity in the slower component (19 S) and the 12 S subunit was 95.3% and 4.7% in the untreated labeled thyroglobulin and 4.0 and 96% in the SDS-treated protein. Migration from left to right.

loid material into the medium in conditions where the cells mostly remain intact thus avoiding contamination of thyroglobulin by intracellular proteases. ^{125}I-thyroglobulin purified from follicles formed in culture in the absence of stable iodide (poorly-iodinated thyroglobulin) shows a single radioactive peak by electrophoresis in polyacrylamide gels. After treatment with 1.5 mM SDS it dissociates almost completely into a faster component migrating at the same rate as the marker 12 S subunit of thyroglobulin (fig.3). This is in agreement with the well-known ability of non-iodinated or poorly-iodinated thyroglobulin to fully dissociate into 12 S subunits in alkaline conditions [13] and in the presence of SDS [10, 14, 15]. In contrast, ^{125}I-thyroglobulin purified from follicles cultured in the presence of stable iodide (iodine-rich thyroglobulin) is incompletely dissociated into subunits even after treatment with SDS (fig.4).

After reduction and electrophoresis in SDS-gels, poorly-iodinated thyroglobulin migrated at a slightly slower rate than the unreduced protein (fig.5). No faster moving bands were observed. Addition of carrier unlabeled-thyroglobulin did not modify the elec-

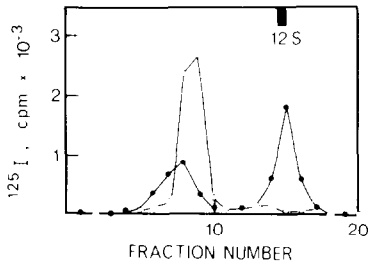

Fig.4. Same experiment as in fig.3 using iodine-rich ^{125}I-thyroglobulin purified from follicles cultured in the presence of 1.5 μM stable iodide in the medium. (○) Untreated protein; (●) after treatment with 1.5 mM SDS. Distribution of radioactivity in the slow migrating peak (19 S) and in the 12 S subunit was 94.8% and 5.2%, respectively, in the untreated protein and, 42% and 58% in the SDS-treated protein.

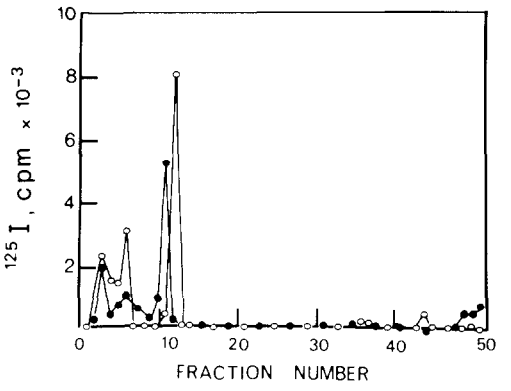

Fig.6. Same experiment as in fig.5 using iodine-rich ^{125}I-thyroglobulin.

trophoretic pattern. In parallel experiments, it was verified by amino acid analysis that, in the conditions of reduction used in these assays, all the disulfide bonds of porcine thyroglobulin were split.

As shown in Fig.6, the unreduced iodine-rich ^{125}I-thyroglobulin did not completely dissociate into subunit. The slower migrating material partially resisted reduction. The same situation was observed with normally iodinated gland thyroglobulin. The persistence of this '19 S' material is likely related to conditions of gel electrophoresis in the presence of SDS and to the presence of iodine in the molecule (unpublished results).

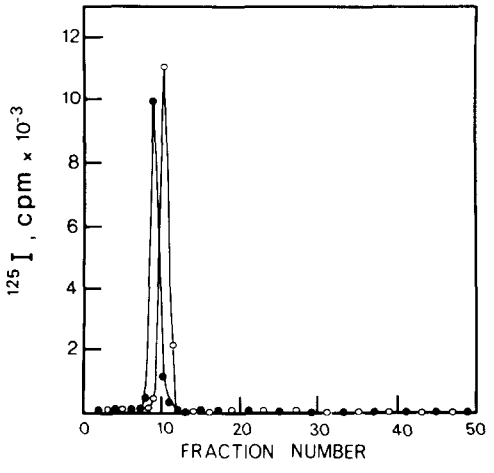

Fig.5. Electrophoresis in SDS-polyacrylamide gels of poorly-iodinated ^{125}I-thyroglobulin. (○) Unreduced protein; (●) after reduction with 1% dithiothreitol in 0.1% SDS 0.1 M sodium phosphate pH 7.2 for 3 h at room temperature or for 5 min at 100°C, before electrophoresis. Migration was for 45 min at 4 mA/tube.

Identical results were obtained with ^{125}I-thyroglobulin from follicles formed in culture in the presence of gelatin without added thyrotropin and pulse-labeled for one hour with ^{125}I (not illustrated).

From the mol. wt. of about 310 000 established by sedimentation equilibrium of reduced and *S*-alkylated gland 19 S thyroglobulin and the almost identical migration in SDS-gel electrophoresis of reduced and unreduced poorly-iodinated ^{125}I-thyroglobulin 12 S subunit purified from thyroid follicles in culture, it is concluded that the 12 S subunit of porcine 19 S thyroglobulin is formed of a single polypeptide chain. The same results were obtained with sheep thyroglobulin. The identity or the non-identity of the 2 chains contained in thyroglobulin remains to the determined. Thus thyroglobulin is formed of the largest polypeptide chain(s) known at the present time.

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