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Isolation and Properties of a Native Subunit of Lamprey Thyroglobulin*

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SUMMARY

The thyroglobulin-like iodoproteins from lamprey thyroid tissue were prepared by repeated salting out between 1.4 and 1.8 M ammonium sulfate. Velocity ultracentrifugation showed only two boundaries, the sedimentation coefficients of which were about 5 S and 12 S. Sucrose gradient centrifugation of the same preparation, pulse-labeled with both ^{125}I and ^{131}I (7 days and 1 hour, respectively before killing), indicated the presence of a third component (17 S) which had a very rapid turnover and corresponds to native thyroglobulin of higher vertebrates. The 12 S component was obtained in ultracentrifugally homogeneous form with a sedimentation constant ($s^0_{20,w}$) and molecular weight of 11.7 and 331,000, respectively. The iodine content was 0.003%.

Lamprey 12 S thyroid protein, the first subunit of thyroglobulin which has been isolated in a pure form, is a native, stable protein with a molecular size corresponding to one-half that of the parent molecule, thyroglobulin.

The ultracentrifugal pattern of the soluble thyroid iodoproteins is very similar in all vertebrates so far studied (1, 2). The major ultracentrifugal component in all species is 19 S thyroglobulin,[1] comprising from 80 to 100% of the thyroglobulin-like iodoproteins. The recently isolated 27 S iodoprotein (4) is a polymer of the subunits (5) found in thyroglobulin, and is present in all of the species examined except in the *Equidae* (1, 2). A slower sedimenting component, with a sedimentation constant close to 12 S, has been found, among mammals, in the guinea pig (2, 6) and the garden dormouse (6, 7), whereas it is fairly common in some poikilotherms (1, 6). A similar ultracentrifugal component (12 S) is formed by the dissociation of thyroglobulin (8), and, according to Edelhoch (9), represents a subunit of 19 S, corresponding to one-half of the parent molecule. However, neither the stable 12 S component nor the dissociation product of thyroglobulin has ever been isolated in a pure form. It has been observed during the course of the present work that a slowly sedimenting species represents the major ultracentrifugal component of the soluble extract from the thyroid gland of the lamprey. This component has been isolated in ultracentrifugally homogeneous form and characterized.

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[1] By definition, thyroglobulin is the soluble thyroid iodoprotein having a sedimentation constant close to 19 S (3). Other thyroid iodoproteins and their subunits will be referred to by their sedimentation constants.

METHODS

Adult lampreys (*Petromyzon fluviatilis* L.), weighing 150 to 200 g each, were caught at springtime while descending the Volturno River and kept in running water at 16°.

Carrier-free, ^{125}I- and ^{131}I-labeled-NaI was injected into the celomic cavity: each lamprey received 25 μC of ^{125}I and 50 μC of ^{131}I, 7 days and 1 hour before death, respectively. The animals were anesthetized with ether and then killed by exsanguination. Since thyroid follicles are scattered in a ventral area between the first and the seventh branchial clefts, this region, which contains all the thyroid-bound radioactivity, was frozen and thawed twice, then minced and sliced. The tissue (approximately 12 g, fresh weight, from 32 animals) was extracted twice with equal volumes of 0.1 M KCl by shaking for 15 hours at 4°. The crude extract, obtained after centrifugation for 10 min at 30,000 × *g* at 4°, was further centrifuged for 30 min at 105,000 × *g* in order to remove subcellular particles, and then submitted to repeated (seven times) salting out between 1.4 and 1.8 M $(NH_4)_2SO_4$ at pH 6.8 and 4°. By exhaustive dialysis against 0.1 M KCl-0.02 M sodium phosphate buffer, pH 7.4, the inorganic radioactive iodides ($^{125}I^-$ and $^{131}I^-$), which are present in large amounts in the crude extract, were completely removed. About 70% of the organic ^{125}I was recovered in the purified extract.

Ultracentrifugation in a linear density gradient was carried out as described previously (10). For analytical purposes a gradient of 5 to 20% (w/v) sucrose was used in the SW 39 rotor of the Spinco model L2-HV centrifuge. For preparative purposes a gradient of 5 to 40% (w/v) sucrose was used in the SW 25.2 rotor; total protein in each centrifuge tube was less than 20 mg, and the equivalent time of centrifugation at 24,000 rpm was about 30 hours. The sucrose gradient fractions were then assayed for protein concentration and radioactivity (see below).

Approximate sedimentation coefficients (s) of the components separated by density gradient were calculated as follows.

$$s = \frac{\text{fraction number of peak} \times 19}{\text{fraction number of rat thyroglobulin peak}}$$

Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge. Double sector cells were used at rotor speeds of 52,640 or 56,100 rpm. The relative proportion of the various ultracentrifugal components was calculated from the relative areas of each schlieren peak without correction for the Johnston-Ogston effect or for the radial dilution of the protein. The sedimentation coefficients were determined by standard procedures; the sedimentation constant was obtained by extrapolation of a plot of sedimentation coefficient with respect to concentration to infinite dilution.

The molecular weight was determined on an ultracentrifugally homogeneous preparation by the method of sedimentation equilibrium according to the meniscus depletion technique of Yphantis (11); the sedimentation equilibrium data were obtained by Rayleigh interference optics. The protein solution (0.04%) was centrifuged at 12,590 rpm at 20° for 24 hours. Fringe displacements (ln c) were plotted against the square of the comparator x-coordinate, and the slopes were used for the calculation of the molecular weight according to the formula

$$M = \frac{2RT\, d \ln c}{(1 - \bar{v}\rho)\omega^2\, dx^2}$$

Since thyroglobulin with an iodine content of 1% has a partial specific volume ($\bar{v}$) of 0.713 (9), a value of 0.73 was calculated for $\bar{v}$ of the poorly iodinated lamprey 12 S protein. A value of 5 g per ml was assumed for the density of iodine.

Ultraviolet spectra were obtained with a recording Beckman DK2 spectrophotometer. The protein concentration was determined by measuring the absorbance at 280 mμ and 210 mμ in a Beckman DU spectrophotometer. At 210 mμ most proteins, including mammalian iodoproteins (4), have nearly the same extinction coefficients, which range from 198 to 215 for a 1% solution (12). Assuming for the lamprey 12 S protein $E^{1\%}_{1cm} = 205$ at 210 mμ, then $E^{1\%}_{1cm}$ is equal to 8.8 at 280 mμ. These values have been used throughout.

The iodoamino acid content in unhydrolyzed protein was determined by spectrophotometric titration according to the method of Edelhoch (13).

Radioactivity was measured in a well-type scintillation counter equipped with a three-channel spectrometer (counting error was kept below 2%).

Stable iodine determinations were performed in triplicate by the Boston Medical Laboratories according to a modified Zak procedure (14).

RESULTS

Ultracentrifugal Analysis of Purified Soluble Thyroid Extract—Two preparations of soluble iodoproteins have been obtained by the $(NH_4)_2SO_4$ salting out technique (see "Methods"). An ultracentrifugal pattern is shown in Fig. 1, which shows two boundaries. The sedimentation rate of the slower peak is approximately 5.4 S, whereas the faster peak has a sedimentation coefficient of approximately 10 S. The proportions of the two components calculated from the areas under the schlieren peaks are 32 and 68%, respectively.

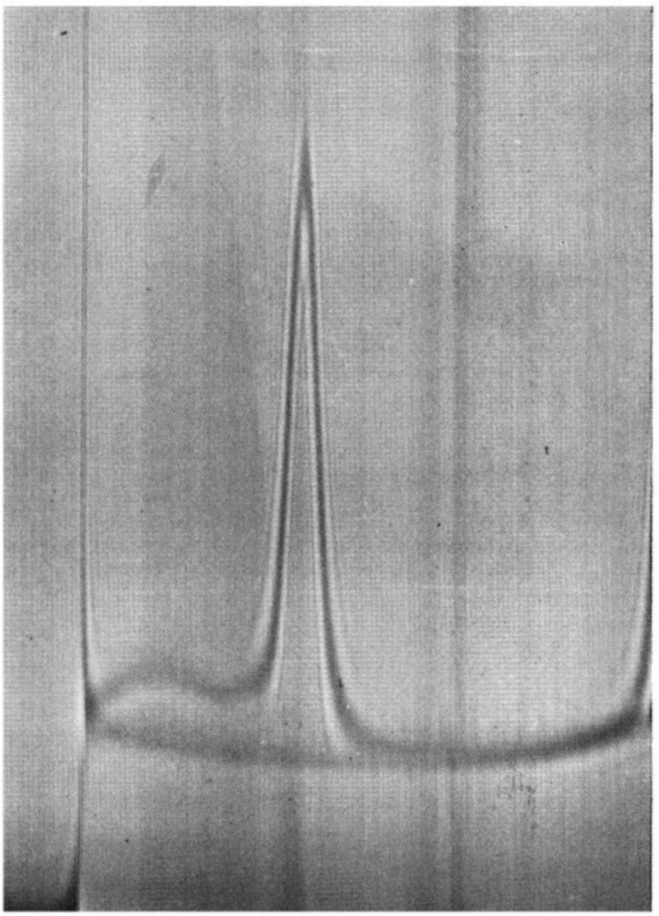

FIG. 1. Ultracentrifugal analysis of lamprey thyroid extract purified by repeated (seven times) salting out between 1.4 and 1.8 M $(NH_4)_2SO_4$. The photograph was taken after 36 min at 52,640 rpm in the Spinco model E centrifuge at 20°; bar angle, 60°; double sector cell; protein concentration, 1% in 0.1 M KCl-0.02 M phosphate buffer, pH 7.4.

The sucrose gradient analysis of the same preparation is reported in Fig. 2. The absorbance pattern is similar to that observed by analytical ultracentrifugation (Fig. 1): two peaks, with sedimentation coefficients of approximately 5 S and 12 S, are clearly evident. The ^{125}I (injected 7 days before killing) pattern is more complicated. Besides the two peaks corresponding to the ultracentrifugal protein components, a third, faster sedimenting peak is present and accounts for 36% of the total radioactivity. The sedimentation coefficient of this peak is about 17 S. The ^{131}I, which was injected 1 hour before killing, is localized almost entirely in the 17 S peak, whereas less than 5% of the radioactivity is associated with the 12 S component.

Isolation and Molecular Properties of 12 S Iodoprotein—The bulk of the purified thyroid extract (20.5 mg) was submitted to preparative density gradient centrifugation (5 to 40% sucrose gradient). The distribution of radioactivity (^{125}I and ^{131}I) and 280 mμ-absorbing material was identical with that shown in Fig. 2. The fractions corresponding to the intermediate peak (21 through 28) were pooled, concentrated, and dialyzed against standard buffer in order to remove the sucrose. A yield of about 80% was obtained, calculated on the basis of the 12 S protein present in the original extract. The ultracentrifugal analysis

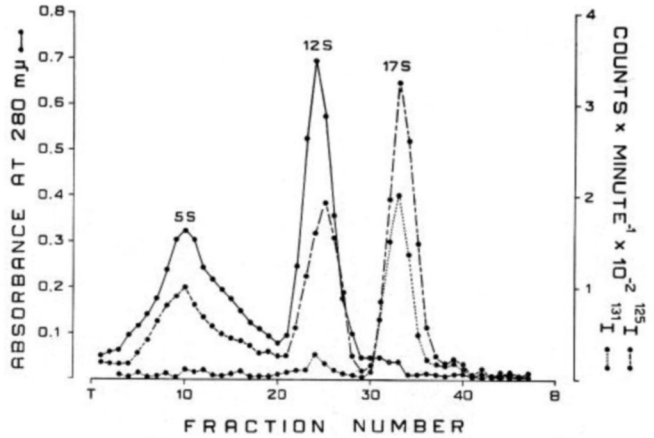

FIG. 2. Sucrose density gradient ultracentrifugation pattern of lamprey thyroid purified extract labeled *in vivo* (^{125}I and ^{131}I injected 7 days and 1 hour before killing, respectively). Sucrose gradient, 5 to 40%; rotor, SW 25.2; equivalent time of centrifugation, approximately 30 hours at 24,000 rpm; total protein, 20.6 mg. *T* and *B* on the *abscissa* indicate top and bottom fraction, respectively.

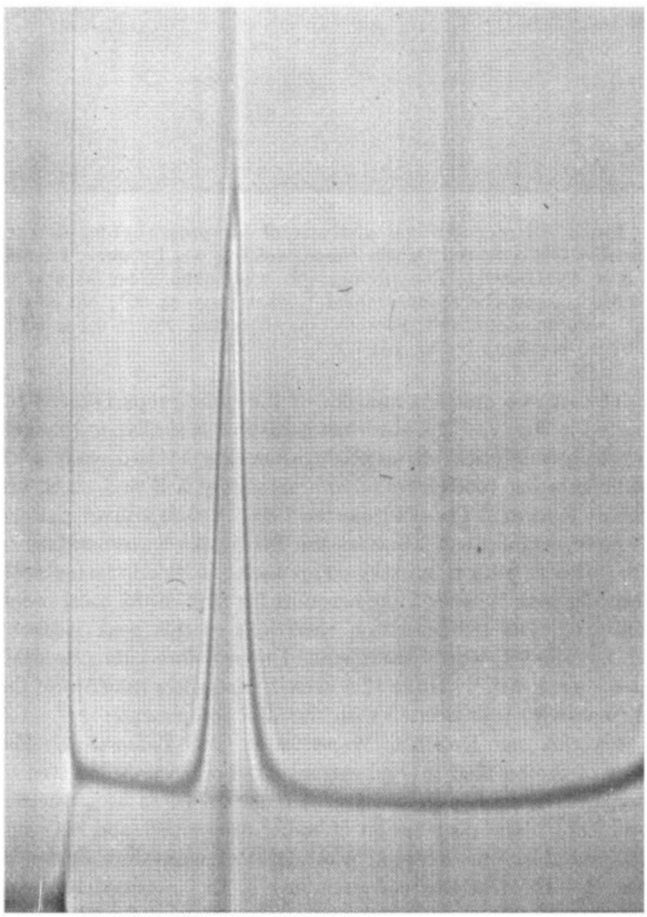

FIG. 3. Ultracentrifugal analysis of purified lamprey 12 S iodoprotein. Conditions were the same as in Fig. 1.

of this preparation showed the presence of a single symmetrical peak (Fig. 3). The concentration dependence of the sedimentation coefficient of this protein is illustrated in Fig. 4. The sedimentation coefficient at infinite dilution ($s_{20,w}$) is 11.75.

The molecular weight was determined by sedimentation equilibrium in three different experiments; values of 313,000, 350,000, and 332,000 were obtained (mean value, 331,000). Data from one of the three experiments are reported in Fig. 5.

The stable iodine content (^{127}I) of the 12 S protein was found to be extremely low; a mean value of 0.003% (average of triplicate determinations) was obtained. However, the 12 S protein contains radioiodine (^{125}I) which was given 7 days before killing; the specific activity of the iodine (^{125}I:^{127}I) is very high (18,000 cpm per μg of iodine).

The ultraviolet spectrum of the 12 S protein shows a maximum absorption near 276 mμ, and the ratio A_{280}:A_{260} is equal to 1.4. The molar extinction coefficient is 2.91 × 10^5 M^{-1} cm^{-1} at 280 mμ and 3 × 10^5 at 276 mμ. The comparison of the ultraviolet

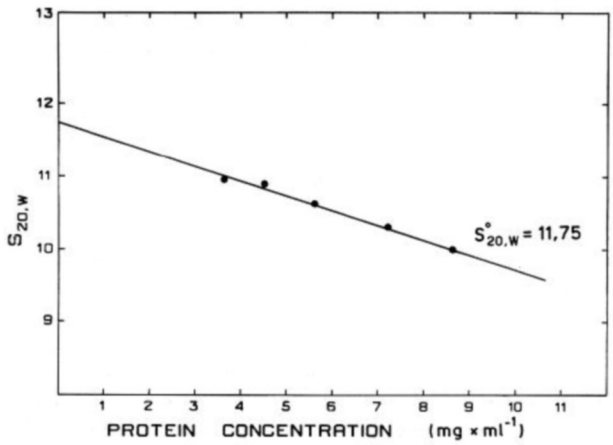

FIG. 4. Dependence of sedimentation coefficient on protein concentration for lamprey 12 S iodoprotein.

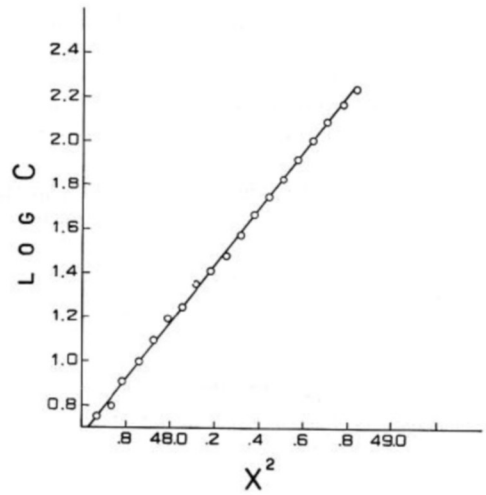

FIG. 5. Sedimentation equilibrium of purified lamprey 12 S iodoprotein. Yphantis cell; rotor speed, 12,590 rpm; equilibrium time, 23 hours; column height, 2.7 mm; temperature, 20°; buffer, same as in Fig. 1. *Abscissa*, square of distance from center of rotation (X); *ordinate*, logarithm of protein concentration (c). The molecular weight obtained from this experiment is 313,000.

spectrum with that of a mammalian 19 S thyroglobulin shows a close similarity except for the region above 300 mμ, where the iodoamino acids absorb. Actually, the spectrophotometric titration showed that iodoamino acids are present in the 12 S component in no more than trace amounts.

DISCUSSION

A hitherto undescribed protein has now been obtained in an ultracentrifugally homogeneous form from lamprey thyroid follicles. This 12 S protein must be considered as a thyroid iodoprotein since it incorporates an important proportion of radioactive iodine (^{125}I) given as a pulse label 7 days before death. Furthermore, its solubility properties in neutral salt solutions are identical with those of mammalian thyroglobulin-like iodoproteins.

An ultracentrifugal component with a sedimentation coefficient close to 12 S has been found among the dissociation products of bovine (15) and rat (5) 19 S thyroglobulin and has been shown to be an intermediate in the biosynthesis of 19 S (16–18). However, neither the intermediate of thyroglobulin synthesis nor the dissociation product of 19 S has ever been isolated in a pure form. On the basis of diffusion and light scattering measurements, Edelhoch (9) has postulated that the 12 S component is a subunit of thyroglobulin and corresponds to one-half of the 19 S molecule. The newly isolated, stable iodoprotein from the lamprey thyroid gland is the only 12 S iodoprotein for which the molecular weight has been directly determined. The molecular weight (331,000) and the values of the other hydrodynamic parameters now obtained for the lamprey 12 S protein agree very well with the values postulated by Edelhoch (9) for the 12 S molecule derived by dissociation of bovine thryoglobulin (Table I).

The lamprey 12 S iodoprotein has to be considered as a native subunit of a heavier ultracentrifugal component resembling thyroglobulin of higher vertebrates. This heavier component exists as a protein constituent of lamprey thyroid extract in minute amounts, being detected only by the radioiodine peak in sucrose gradient analysis (Fig. 2). Because of its extremely low concentration in the thyroid extract, it was not possible to isolate the heavier protein and assess its molecular weight. Its sedimentation coefficient, as calculated from the sucrose gradient pattern, is equal to 17 S, a value which is compatible with that of a dimer of the 11.7 S protein. In this case, the molecular weight of 17 S lamprey iodoprotein would be 662,000, a value which is very close to that of the 19 S thyroglobulin of mammals.

Besides the 12 S protein, purified lamprey thyroid extract contains a slower ultracentrifugal component with a sedimentation rate of approximately 5 S (Figs. 1 and 2). No detectable amounts of stable (^{127}I) iodine were found in this isolated fraction, but it incorporates radioiodine (^{125}I) and has the same solubility properties as other thyroglobulin-like proteins; *i.e.* it precipitates between 1.4 and 1.8 M ammonium sulfate. It is possible that such a light component is a subunit of 12 S, corresponding to the 6 S found by Vecchio *et al.* (5) from the dissociation of the 27 S iodoprotein and also reported after reduction of the interchain disulfide bonds of bovine thyroglobulin (15). A 5 S component also corresponds to the well defined 3 to 8 S fraction of crude extracts of thyroid slices incubated with labeled amino acids (16–18). In addition, it has been recently found in purified thyroid extracts of rats and guinea pigs labeled *in vivo* with ^{3}H-leucine (20).

Lamprey thyroid gland appears to contain the full spectrum of thyroglobulin precursors, including the 5 S and the 12 S species. The reasons for the existence of these two precursors as stable proteins in the lamprey and their absence in most mammals are not yet known. Differences in the structure of the basic subunits, or a slower rate of polymerization to 17 S, could explain these findings. Actually, the iodination of 5 S and 12 S lamprey proteins seems to be much slower than that of thyroglobulin, since 1 hour after the administration of radioiodine (^{131}I) the 17 S is practically the only species which is labeled. On the contrary, after a 7-day pulse label of ^{125}I *in vivo*, more than 60% of the radioiodine is incorporated in the 5 S and 12 S species. The presence in lamprey thyroid of native, stable subunits results in their iodination, whereas radioiodine is not incorporated into subunits when the rate of their polymerization is so high that they do not accumulate as stable proteins, as appears to be the case in most mammalian species.

It is remarkable, nevertheless, that the molecular organization of thyroid proteins is very similar throughout all classes of vertebrates. The polymerization of subunits leads to the formation of well defined proteins of increasing molecular size, such as 12 S, 19 S, and 27 S. Thyroglobulin (19 S) is the major stable component in all thyroid extracts, except in the lamprey. However, iodination takes place mainly on thyroglobulin in all species, including the lamprey. The apparent absence of a stable heavier iodoprotein in the lamprey can be explained by its rapid turnover from thyroid follicles. In most higher vertebrates, the 12 S appears to dimerize to 19 S very rapidly and therefore does not accumulate in the gland. On the contrary, in lower vertebrates, a stable 12 S protein is fairly common, even though it is a minor component of thyroid extract. In the lamprey, which belongs to the most primitive class of vertebrates, and in which differentiated thyroid follicles appear phylogenetically, the 12 S native thyroglobulin subunit represents the major thyroid protein.

TABLE I

Hydrodynamic properties of lamprey 12 S iodoprotein compared to those reported by Edelhoch (9) for bovine thyroglobulin (19 S) and its dissociated 12 S subunit

Property	Bovine 19 S	Bovine dissociated 12 S[a]	Lamprey native 12 S
$s^0_{20,w}$	19.4	12.1	11.7
$D_{20,w} \times 10^7$	2.49	3.11	3.11
Mol. wt.	669,000	335,000	331,000
f/f_0	1.49	1.51	1.49
a/b[b]	9.0	9.4	9.0

[a] For this component the sedimentation coefficient was measured, the molecular weight was assumed (half of 19 S), and the other values were calculated on the basis of molecular weight.

[b] Axial ratio for a prolate ellipsoid of revolution.

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