ASSOCIATION OF ISOLATED LABELED SUBUNITS OF THYROGLOBULIN*

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When radioactive amino acids are incorporated into TG** (19S) by thyroid slices, label is detected in more slowly sedimenting proteins (3-8S and 12S) before it appears in TG (Seed and Goldberg, 1963, 1965, and Lissitzky et al. 1964). Edelhoch (1960, 1964) has shown that TG is composed of two half-molecules (12S) which are, to a considerable extent, non-covalently linked, and which, in turn, consist of two polypeptide chains (3-6S) which are linked by disulfide bonds. This suggested that the more slowly sedimenting radioactive proteins are subunits of TG and possibly represent intermediate stages in TG biosynthesis. This postulate is supported by the results of kinetic experiments and by the physical and immunologic properties of these proteins (Seed and Goldberg, 1963, 1965, Lissitzky et al. 1964, and Sellin and Goldberg, 1965). Newly made TG has some properties which differ from those of preformed TG (Seed and Goldberg, 1963, 1965, Lissitzky et al. 1964, and Sellin and Goldberg, 1965). TG into which radioactive amino acids or iodine have been incorporated sediments more slowly† and is more labile to dissociation to 12S half-molecules than pre-existing TG. Further, iodine-labeled protein is more stable to dissociation by detergent than amino acid-labeled (and presumably poorly iodinated or uniodinated) protein (Sellin and Goldberg, 1965). Since iodination occurs after formation of the polypeptide backbone (Goldberg et al. 1964, Taurog and Howells, 1964,

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** The abbreviations used are: TG, thyroglobulin, SDS, sodium dodecylsulfate; PBS, phosphate buffered saline, 0.15 M sodium chloride in 0.01 M potassium phosphate, pH 6.8.

† The peak of radioactivity (using ^{125}I or ^{14}C-amino acid in the "19S region" of a sucrose gradient does not coincide precisely with the optical density pattern at 280 mu. If the optical density peak is taken as 19S, the peak of ^{125}I-labeled protein is 18.5S and of ^{14}C-amino acid-labeled protein is 17.5-18S (Seed and Goldberg, 1963, 1965).

Soodak et al. 1964, Lissitzky et al. 1964, Seed and Goldberg, 1965, and Tishler and Ingbar, 1965), the greater ease of dissociation of the amino acid-labeled TG was ascribed to its less compact tertiary structure, a manifestation of its relative biological immaturity. The chemical alterations which confer "maturity" upon TG may be several, including iodination, carbohydrate addition, and possibly interchain co-valent bond formation.

This report will show that the more slowly sedimenting amino acid-labeled thyroid proteins after purification can be associated to 19S thyroglobulin. Association occurs upon incubation in the presence of carrier TG but is increased under conditions of chemical iodination. Chemical iodination also increases the sedimentation of the amino acid-labeled TG to a position which coincides with the 19S protein optical density peak, and leads to the formation of a labeled 27S protein. The chemically modified TG is much more resistant to dissociation to its half-molecule by agents which disrupt non-covalent bonds than its untreated control. These findings may reflect the biological processes which lead to TG formation and maturation, and provide further support for the concept that both labeled 12S and 3-8S proteins are subunits of TG.

Experimental: The methods of thyroid slice incubations and of sucrose gradient centrifugation of thyroid proteins are as previously described (Seed and Goldberg, 1963, 1965, Sellin and Goldberg, 1965), except that the SW 25.1 rotor was used in the Spinco model L-2 ultracentrifuge, and the optical density at 280 mu was recorded automatically in the Gilford Absorbance Recorder.

Chemical iodination of thyroid proteins was carried out by a modification of the method of Edelhoch (1962). When ^{14}C-and ^{3}H-labeled 12S and 3-8S proteins were to be chemically iodinated, carrier TG was added to a concentration of 0.5%. Chemical iodinations were performed at pH 6.8 in PBS with an amount of I_2 equivalent to the molar concentration of tyrosine in TG. Under the conditions used here, iodination should proceed to a very limited extent and, therefore, no significant change in the yellow color of the reaction occurred during incubation. Prior to gradient analysis most of the yellow color was removed by dialy-

sis against PBS. Pure fractions of labeled thyroid proteins (19S, 12S, and 3-8S) were prepared by pooling the appropriately sedimenting fractions of the sucrose gradient, adding carrier TG, and precipitating with 50% ammonium sulfate. A 35-45% ammonium sulfate fraction of calf thyroid soluble proteins was used as "carrier TG". While the major optical density peak at 280 mu in all experiments was assumed to have a sedimentation constant of 19, there were also small amounts of more rapidly (27S) and more slowly (3-8S and 12S) sedimenting proteins.

Results: Conversion of labeled 12S protein to TG-like protein. When amino acid-labeled 12S protein prepared with rat thyroid slices was incubated in the presence

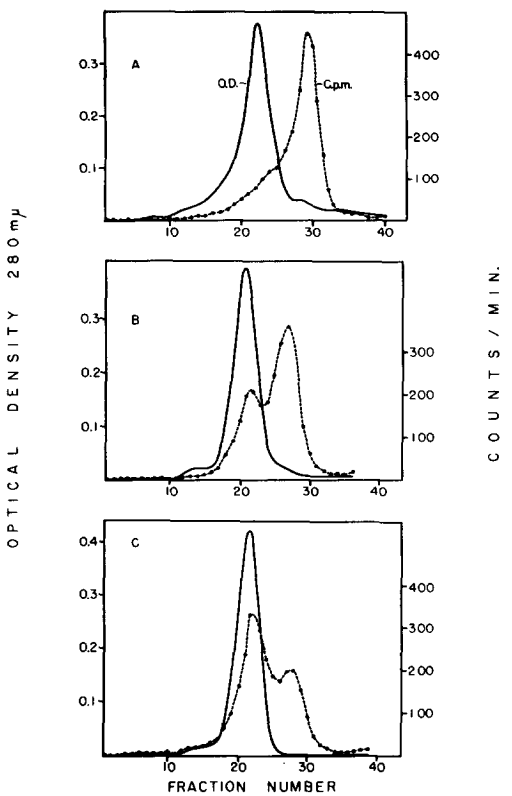

Fig. 1. Conversion of labeled 12S protein into TG-like protein. Purified ^{14}C-leucine-labeled rat 12S protein containing carrier calf TG was taken up in 3 ml. of PBS and divided into 3 equal fractions, each of which was kept at (A) 0° for 17 hours, (B) 24° for 3 hours, then 4° for 14 hours, or (C) 35° for 17 hours. Each fraction was then layered on 5-20% sucrose in PBS for centrifugation at 23,000 RPM for 24 hours. In (A) the major radioactive peak sediments at 12S and the major optical density peak at 19S.

of carrier TG, there was a slow conversion of label to a more rapidly sedimenting form (Fig. 1). The association proceeded more rapidly at 35° than 24°. The main peak of radioactivity of the newly formed material sedimented at about 18S, slightly slower than the calf TG optical density peak. In addition, as seen in Fig. 1C, incubation at 35° has led to the appearance of radioactivity in the 27S region of the gradient.

Chemical iodination of labeled 12S protein. When labeled rat 12S protein was treated with the iodine reagent in the presence of carrier calf TG, there was a significant conversion of the 12S label to a more rapidly sedimenting species (Fig. 2). This conversion was much more extensive and the labeled protein was more nearly superimposable on the 19S protein peak when compared with a control sample which was not so treated.

Conversion of labeled 3-8S protein to TG with and without chemical iodination. The spontaneous conversion of labeled calf 3-8S protein to a form sedimenting with TG was found when the incubation was carried out in the presence of carrier

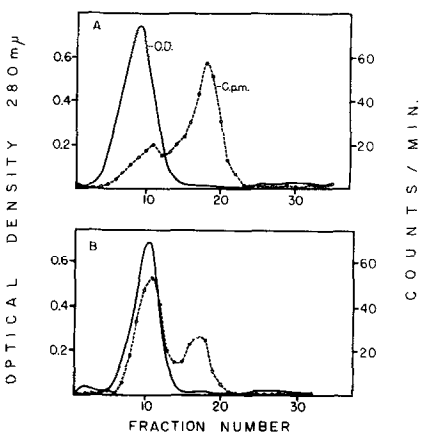

Fig. 2 Effect of chemical iodination on labeled 12S protein. Purified ^{3}H-leucine-labeled rat 12S protein containing carrier calf TG was taken up in 2 ml. of PBS. (A) One ml. was kept at 24° for 2 hours and at 4° for 15 hours. (B) To one ml. was added 0.025 ml. of 0.04 M iodine in 0.48 M KI. After 2 hours at 24°, the solution was kept at 4° for 15 hours. Both (A) and (B) were then dialyzed against cold PBS prior to analysis as described in Fig. 1. The major radioactive peak in (A) sediments at 12S.

calf TG. The conversion of the 3-8S labeled protein appeared to be slower than the conversion of the 12S labeled protein to TG. This association was much more extensive under the conditions of chemical iodination. As shown in Fig. 3, after treatment radioactivity sediments slightly faster than the 19S optical density peak. This phenomenon has been more obvious in other experiments and may be due to the greater reactivity to iodination of tyrosine residues in the unfolded quarter-molecule than in the native TG (Edelhoch, 1964).

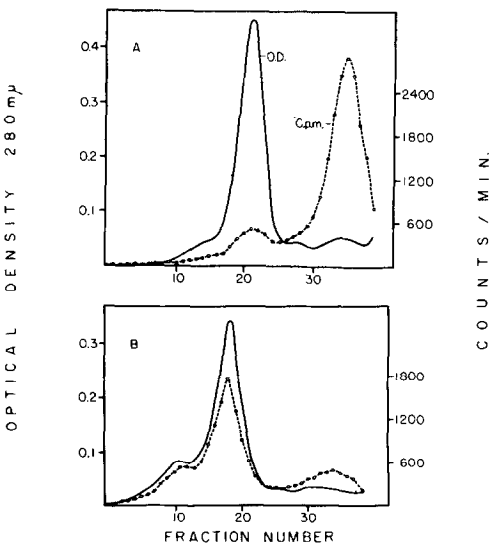

Fig. 3. Effect of chemical iodination on labeled 3-8S protein. Purified ^{14}C-lysine and ^{14}C-arginine-labeled calf 3-8S protein containing carrier calf TG was taken up in 2 ml. of PBS. (A) was treated as (A) in Fig. 2 and (B) was treated with the iodine reagent as (B) in Fig. 2. Centrifugation was at 23,000 RPM for 24 hours. The major radioactive peak in (A) sediments at 4S.

Use of chemical iodination to increase the sedimentation of labeled TG. As seen in Fig. 4A and B, chemical iodination of labeled TG shifts the sedimentation of the radioactivity peak so that it coincides with the 19S optical density peak. Similarly, radioactivity is now associated with the increased 27S protein peak.

Inability of SDS to dissociate chemically iodinated TG to 12S. It was found that 0.0015 M SDS which readily dissociates TG to its half-molecule (radioactivity

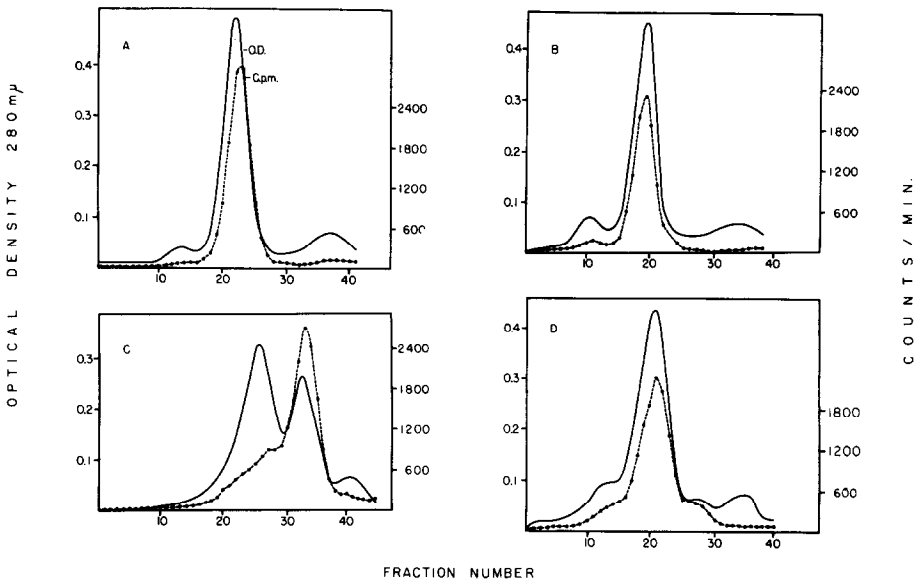

Fig. 4. Effect of chemical iodination on the sedimentation and dissociation of labeled TG. Purified ^{14}C-lysine and ^{14}C-arginine-labeled calf TG were taken up in 4 ml. of PBS. Two ml. (A and C) were treated as (A) in Fig. 2 and 2 ml. (B and D) were treated as (B) in Fig. 2, except that 0.05 ml. of the iodine reagent was used. Following dialysis against cold PBS, each sample was divided in two and 1 ml. of each (C and D) was treated with 0.0015 M SDS at 24° for 30 minutes before centrifugation in 5-20% sucrose in PBS saturated at 4° with SDS. One ml. of the untreated sample (A) and 1 ml. of the iodinated sample (B) were analyzed as in Fig. 1. Centrifugation was at 23,000 RPM for 25 hours for (C)and (D).

to a greater extent than optical density) (Fig. 4C) is relatively ineffective with the chemically iodinated material (Fig. 4D). Furthermore, the 27S protein persists to a considerable extent in the presence of the SDS. Similarly, treatment of native calf 27S protein with SDS leads to formation of relatively small amounts of 19S and 12S particles with the major fraction remaining as 27S or its unfolded form, 23-24S.

Discussion: Edelhoch et al. (1965) have recently reported on the spontaneous aggregation to 12S and 19S molecules upon oxidation of chemically reduced TG subunits (3S). It is possible that a similar mechanism is involved in the association

of labeled 3-8S subunits during incubation in the presence of carrier TG. Another mechanism which must be considered is one involving disulfide exchange between the labeled units and the unlabeled ones of the carrier calf TG to form hybrid 19S molecules.

Presumably disulfide bond formation is not required for 12S dimerization. Conversion of labeled 12S protein to an 18S particle may proceed by way of an exchange reaction with unlabeled 12S units in equilibrium with the 19S dimer. Because of concentration considerations, this type of hybridization may be more favored than the direct association of labeled 12S units with each other. Furthermore, other experiments indicate that 16S rather than 18S particles are the predominant aggregates formed by association of labeled rat 12S units with each other. In thyroid slices from normal rats or rats treated with Tapazole most of the amino acid label (and optical density in the latter case) appears in the 16S and 12S regions, even when pulse-labeled slices are further incubated in the presence of unlabeled amino acid. These data suggest that in the rat under conditions not favorable for iodination, or thyronine formation, there is a block in association of subunits to 19S protein with an accumulation of 12S protein and it asymmetrical 16S dimer. It might then be expected that if the isolated rat 12S units come together with one another, 16S particles would be produced rather than the more rapidly sedimenting ones found here.

Other data suggest that carrier TG is important for the association reaction. Whether this is due solely to its contribution of hybridizing subunits or because of some other protein (enzyme) or factor which accelerates the conversion remains to be seen. That non-covalent bonds are involved in the 12S association is indicated by the ease of dissociation of the labeled aggregate with SDS.

Although the conditions of chemical iodination used here are mild, one is not able at this point to attribute the described aggregation and altered stability and sedimentation properties of both the labeled and carrier proteins solely to the partial iodination of protein tyrosines. Oxidative side reactions complicate the mildest of iodinating conditions (Ramachandran, 1956). Disulfide bond forma-

tion or thyronine formation between 12S units could account for the inability of agents which disrupt non-covalent bonds to bring about dissociation of the aggregate, and these may be mechanisms whereby TG maturity is achieved. The well known inability of thyroid slices to form significant amounts of thyronine may contribute to the difference in sedimentation properties of the preformed and newly made TG. It is of interest in this regard that there is a fraction of native 19S TG which cannot be dissociated by detergent (Edelhoch and Lippoldt, 1960, Sellin and Goldberg, 1965). This suggests that covalent bonds might be involved in holding the two 12S units together in such a fraction. Similar considerations may be invoked in explaining the resistance to dissociation of the labeled 27S protein formed during treatment with the iodine reagent.

While it has been shown that there is an association of subunits under conditions of chemical iodination, the data do not indicate at what level of aggregation such treatment exerts this effect. In fact, other data indicate that aggregation, at least up to the point of a 16S particle in the rat and a 17.5S particle in the sheep and calf, may proceed in the complete absence of biological iodination but that conformational or structural changes leading to more rapidly sedimenting and more stable particles occur upon or after iodination. In such a case iodination and other reactions may act to "pull" the equilibrium in favor of the more rapidly sedimenting particle.

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