Formation of Dehydroalanine Residues during Thyroid Hormone Synthesis in Thyroglobulin*

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The formation of dehydroalanine residues during thyroid hormone synthesis in thyroglobulin catalyzed by thyroid peroxidase was studied by two methods.

1. Human goiter thyroglobulin (0.04% I) was iodinated to ~60 atoms of I using iodide and thyroid peroxidase. This led to the formation of 2.9 thyroid hormone (thyroxine and triiodothyronine) residues/mol. Dehydroalanine residues, formed simultaneously, were characterized and quantitated as S-benzylcysteine after reaction of the iodinated thyroglobulin with benzyl mercaptan, followed by acid hydrolysis and amino acid analysis. About 1 benzylcysteine residue was found for each hormone residue formed.

2. [U-14C]Tyrosine-labeled iodine-free hog thyroglobulin was prepared by incubation of hog thyroid slices with [U-14C]tyrosine. Enzymatic iodination of this thyroglobulin led to the formation of 2.8 [14C]dehydroalanine residues/mol. The latter were characterized and quantitated as [14C]alanine after treatment of the iodinated thyroglobulin with sodium borohydride, followed by acid hydrolysis. For each hormone residue synthesized during enzymatic iodination, about 1 alanine residue was formed. When [U-14C]tyrosine-labeled hog thyroglobulin was iodinated and then hydrolyzed without borohydride treatment, the [14C]dehydroalanine residues appeared in the hydrolysate as [14C]acetic acid resulting from decarboxylation, during acid hydrolysis, of initially formed [14C]pyruvic acid. Acid hydrolysis also converted [14C]-hormone residues to [14C]-thyronine by deiodination. For each hormone residue synthesized during enzymatic iodination, about 1 molecule of labeled acetic acid was found.

S-Benzylcysteine, labeled acetic acid, and labeled alanine in the various acid hydrolysates are derived from the same precursor, dehydroalanine residues. Since the molecular ratios of all these products to the hormone residues formed in the course of enzymatic iodination approach unity, dehydroalanine residues are formed concomitantly with hormone residues in a molar ratio of 1 and thus are the sole product of the alanine side chain of one-half of the iodinated tyrosine residues involved in thyroid hormone synthesis. This side chain is eliminated in the course of the formation of hormone residues, but remains within the polypeptide chain of thyroglobulin.

Thyroglobulin, the principal protein of the thyroid gland, is the storage form of the thyroid hormones thyroxine and triiodothyronine. These iodoamino acids are not incorporated into thyroglobulin on ribosomes like other amino acids. They are formed within the completed polypeptide chain by conversion of some of the tyrosine residues to hormone residues. In the course of this conversion, the former undergo iodination followed by oxidative coupling. The coupling of 2 diiodotyrosine residues in thyroglobulin results in the fission of 1 of them next to its aromatic ring. A review of the mechanism of thyroid hormone synthesis has been published recently (1).

In spite of a number of model reactions carried out over the last 3 to 4 decades, details of the mechanism of the coupling reaction still remain obscure. Several attempts were made (2–6) to determine the fate of the “lost side chain” (2) that remains when the aromatic moiety of the diiodotyrosine residue which undergoes fission is transferred to the other iodothyrosine residue. These experiments were not carried out with thyroglobulin, but with models such as diiodotyrosine (3, 4), derivatives (5) or analogs (2, 5) of diiodotyrosine, acyldiiodotyrosine peptides (5), or iodinated polypeptide (6). In many of these nonenzymatic model reactions, nonphysiological reaction conditions were used.

We have recently reported (7) a search for the lost side chain in which the natural substrate, thyroglobulin, and thyroid peroxidase were used. This enzyme catalyzes both iodination (8, 9) and coupling (10, 11) in vivo. We found that the lost side chain appears in the enzymatic hydrolysate of thyroglobulin as pyruvic acid (7). We did not determine in which form the lost side chain exists in thyroglobulin before hydrolysis, but we indicated that the pyruvic acid probably is derived from dehydroalanine residues.

We now report experiments which prove that the lost side chain is indeed present in thyroglobulin in the form of dehydroalanine residues. For each hormone residue synthesized, 1 dehydroalanine residue is formed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Benzy1 mercaptan (99%) and S-benzylcysteine were from Aldrich and N-ethylmorpholine (99+%) and sodium borohydride from Sigma. Acetyldidehydroalanine was from ICN (K and K Laboratories Division), guanidine hydrochloride ("ultrapure") from Schwarz/Mann, and constant boiling hydrochloric acid from Pierce. [2,4,6,8,10,12-14C]Acetic acid (sodium salt) was from ICN, and [U-14C]pyruvic acid (sodium salt) and [U-14C]alanine were from Amersham. Human goiter thyroglobulin (0.04% I/mol) was a gift from M. Rolland, Faculté de Médecine, Marseille. [U-14C]Tyrosine-labeled hog thyroglobulin (12) was prepared as described recently (7). Hog thyroid peroxidase was prepared according to Pommier et al. (9).

Buffers—N-Ethylmorpholine/acetic acid buffer was prepared by adjusting the pH of a 10% aqueous solution of N-ethylmorpholine to

1 Dehydroalanine = 2-aminoacrylic acid.
Formation of Dehydroalanine during Thyroid Hormone Synthesis

8.5 with 2 M acetic acid. The slightly opalescent solution was clarified by filtration through a 0.22-μm Millipore filter. Sodium borate/guanidine buffer was prepared by adding guanidine hydrochloride to 0.1 M sodium borate buffer, pH 8.5, to a final guanidine concentration of 6 M. The pH was then readjusted to 8.5 with NaOH.

**Iodination of Thyroglobulin—**Enzymatic iodination of [U-14C]thyroglobulin was carried out according to Meyer et al. (11), using [125I]iodide and thyroid peroxidase in the conditions described by Gavaret et al. (7). Human goiter thyroglobulin (13 mg, 20 nmol) was iodinated in the same manner. The iodinated thyroglobulins were dialyzed against water and then lyophilized. The number of thyroxine and triiodothyronine residues formed in the course of iodination was checked by paper chromatography, after enzymatic hydrolysis (7), in a parallel experiment in which the same thyroglobulin was iodinated similarly, but using [125I]iodide. The solvents 1-butanol/ethanol/0.5 M NH₄OH (5:1:2) and n-pentanol (2-methyl-2-butanol), saturated with NH₄OH (upper phase), were used.

**Reaction of Thyroglobulin with Benzyl Mercaptan—**Thyroglobulin prepared by iodination of 13 mg of human goiter thyroglobulin was dissolved in 200 μl of N-ethylmorpholine/acetic acid buffer and 200 μl of water. Absolute ethanol (700 μl) and benzyl mercaptan (50 μl) were then added. The initially clear solution soon became turbid. The reaction mixture was freed from oxygen by evacuation and then sealed in a nitrogen-filled glass tube. Good mixing was obtained by tumbling at room temperature. After 10 days, the reaction mixture was dialyzed (Visking casing), first several times against 30% ethanol and then against water, with many changes, until most droplets of benzyl mercaptan had disappeared. The retentate was then lyophilized and the residue, after addition of 2 mg of phenol, was hydrolyzed with 2 ml of constant boiling hydrochloric acid for amino acid analysis (see below). A control experiment was carried out in the same manner except that benzyl mercaptan was omitted.

**Reaction of Acetyldehydroalanine and of Iodinated [U-14C]Thyroglobulin with Sodium Borohydride—**Acetyldehydroalanine (I mg, 7.7 μmol) was dissolved in 1 ml of freshly prepared sodium borate/guanidine buffer and the pH readjusted to 8.5 with sodium hydroxide. Sodium borohydride (50 mg) was then added. The mixture was kept at room temperature for 2½ days with occasional slow stirring and then lyophilized. Constant boiling hydrochloric acid (1 ml) was added carefully in order to avoid excessive foaming. The solution was evaporated to dryness and the residue hydrolyzed (see below) with 3 ml of constant boiling hydrochloric acid. A control experiment was carried out in the same manner except that sodium borohydride was omitted.

Iodinated [U-14C]thyroglobulin (3.3 mg, 5 nmol, determined spectrophotometrically at 280 nm; E₁% = 10) was dissolved in 1 ml of freshly prepared sodium borate/guanidine buffer, pH 8.5, and then treated with sodium borohydride (50 mg) as described for acetyldehydroalanine, except that the incubation period was extended to 3 days. During the incubation of thyroglobulin (as well as of acetyldehydroalanine), the initially clear solution became cloudy and finally a precipitate formed. The pH dropped first by nearly 1 pH unit and then slowly rose again to attain a final value of 9.5 to 10. It was then readjusted to 8.5 with hydrochloric acid. After another 5 h, unreacted sodium borohydride was decomposed by careful addition of 1 ml of constant boiling hydrochloric acid and the reaction mixture concentrated in a rotating evaporator to about ½ its volume. Final drying was done by lyophilization. The residue was hydrolyzed with 3 ml of constant boiling hydrochloric acid as described below. A control experiment was carried out in the same manner except that sodium borohydride was omitted.

Since the reduction of dehydroalanine residues in thyroglobulin was performed in 6 M guanidine in order to denature the protein (see below), preliminary experiments with acetyldehydroalanine were also performed in the presence of 6 M guanidine. Six molar guanidine does not interfere with an efficient reduction of the double bond with sodium borohydride. For some unexplained reason, acetylatedalanine yields were even higher in the presence of guanidine than in its absence.

**Acid Hydrolysis—**Acetyldehydroalanine and iodinated thyroglobulin (treated or not treated with benzyl mercaptan or with sodium borohydride) were hydrolyzed with constant boiling hydrochloric acid. Hydrolysis was carried out for 24 h at 105°C in evacuated sealed glass tubes.

**Cation Exchange Chromatography—**Aliquots of the hydrolysates of iodinated human goiter thyroglobulin (1%), treated or not treated with benzyl mercaptan (see above), were injected onto the long column (18.3 cm) of a Beckman 121-M amino acid analyzer. Aliquots of the hydrolysates of acetyldehydroalanine (0.1%), treated or not treated with sodium borohydride (see above), were analyzed in the same manner.

The entire hydrolysate of the sodium borohydride-treated iodinated [U-14C]thyroglobulin and the hydrolysate obtained in the control experiment in which sodium borohydride was omitted were chromatographed, after the addition of 500 μg of carrier L-alanine, on a packed column of a Beckman 121-B amino acid analyzer (---). In a control experiment, the same iodinated thyroglobulin was treated in the same manner except that benzyl mercaptan was omitted (----).

**RESULTS**

Identification and quantitation of dehydroalanine residues in thyroglobulin were achieved by two methods: 1) their conversion to S-benzylcysteine residues by reaction with benzyl mercaptan, and 2) reduction of the double bond with sodium borohydride with formation of alanine residues.

**Reaction of Iodinated Human Goiter Thyroglobulin with Benzyl Mercaptan—**Iodination of human goiter thyroglobulin with thyroid peroxidase for 90 min led to the incorporation in thyroglobulin of ~60 atoms of iodine/mol and to the formation of 2.9 hormone residues/mol (2.6 thyroxine and 0.3 triiodothyronine). Reaction of this thyroglobulin with benzyl mercaptan led to the formation of S-benzylcysteine residues which were identified by amino acid analysis after acid hydrolysis of the sample. In a control experiment carried out without the addition of benzyl mercaptan, no S-benzylcysteine was formed (Fig. 1). When an aliquot of the hydrolysate corresponding to 0.2 nmol of mercaptan-treated thyroglobulin was injected into
the amino acid analyzer, the area under the S-benzylcysteine peak corresponded to 0.5 to 0.6 nmol (2.5 to 3.0 residues/mol of thyroglobulin). Thus, 1 mol of S-benzylcysteine was found for each mole of hormone present in the iodinated thyroglobulin.

Reaction of [U-14C]Thyroglobulin with Sodium Borohydride—Iodination of [U-14C]tyrosine-labeled thyroglobulin with thyroid peroxidase for 90 min led to the formation of 2.8 hormone residues/mol (2.5 thyroxine and 0.3 triiodothyronine).

In order to select reaction conditions suitable for the reduction of dehydroalanine residues by sodium borohydride, preliminary experiments were carried out with acetyldehydroalanine. The reaction conditions described under "Experimental Procedures" led to the formation, after acid hydrolysis, of alanine in 97% yield as determined by amino acid analysis. In a control experiment in which sodium borohydride was omitted, no alanine was detected (figure not shown).

Similar experimental conditions were used for the treatment of iodinated [U-14C]tyrosine-labeled thyroglobulin. Cation exchange chromatography of the acid hydrolysates of iodinated [U-14C]tyrosine-labeled thyroglobulin which had been treated or not treated (control) with sodium borohydride gave the elution profile shown in Fig. 2. After borohydride treatment, 14Cl alanine was easily identified, while in the control experiment, 14Cl acetic acid was present. Pertinent portions of the elution profiles obtained after treatment of the labeled thyroglobulin with sodium borohydride and without such treatment (control) are compared in Fig. 2B. The profile obtained after treatment with borohydride shows a large 14Cl alanine peak and only minimal radioactivity in the acetic acid region, while the opposite is true for the profile observed when treatment with sodium borohydride was omitted. Pyruvic acid, formed after enzymatic hydrolysis (7), was absent after acid hydrolysis. (The retention volumes of acetic and pyruvic acid were checked with authentic standards.) As expected, heating with hydrochloric acid had decarboxylated pyruvic to acetic acid. A comparison of the profile obtained in the control experiment (Fig. 2A) with the profiles previously obtained after enzymatic hydrolysis (Figs. 1 and 2A of Ref. 7) shows that mono- and diiodotyrosine are absent in the acid hydrolysate. Separate column and paper chromatographic experiments revealed that these iodotyrosines had been deiodinated to tyrosine. The comparison further shows that thyroxine and triiodothyronine had similarly been deiodinated to thyronine.

In addition to the major peaks, the elution profile also shows a number of minor peaks. Such minor peaks have been observed in many laboratories after enzymatic hydrolysis of thyroglobulin and usually have been ascribed to peptides resulting from incomplete hydrolysis. Our observation that as many minor peaks are present after acid hydrolysis for 24 h at 105°C casts doubt on such an interpretation. Furthermore, some of the minor peaks observed after enzymatic hydrolysis do not contain iodine (Fig. 2 of Ref. 7) and, therefore, cannot be explained by the assumption that iodinated peptides may be more refractory to hydrolytic splitting than noniodinated ones. Only very few minor peaks were observed in the elution profiles of hydrolysates of newly synthesized iodine-free thyroglobulin (results not shown). Numerous minor peaks were present only after iodination. It must, therefore, be assumed that many and possibly most of the minor peaks are due to oxidation products formed in the course of the iodination with thyroid peroxidase.

Further analysis of the data depicted in Fig. 2 allows a calculation of the stoichiometric relationship between the amount of alanine or acetic acid formed and the number of hormone residues. The number of thyronine residues (2.8 residues/mol of thyroglobulin) was not affected by sodium borohydride. The thyronine/alanine radioactivity ratio was 4.6 and the thyronine/acetic acid ratio in the control experiment was 7.6. This corresponds to molar ratios of 0.92 and 1.0, respectively, since thyronine has 15, alanine 3, and acetic acid 2 labeled carbon atoms. Hence, the acetic acid/alanine molar ratio approaches unity.

Both acetic acid and alanine must be derived from the same precursor which can only be dehydroalanine residues. No other precursor would be reduced to alanine residues by sodium borohydride. Since the thyronine/alanine and thyronine/acetic acid molar ratios, and, consequently, also the hormone residue/dehydroalanine residue molar ratio, approach unity and since the thyronine experiments contained 2.8 hormone residues/mol, the number of dehydroalanine residues must also be 2.8 residues/mol of thyroglobulin. This was corroborated by the fact that addition of benzyl mercaptan to dehydroalanine residues in thyroglobulin led to the formation of 2.5 to 3.0 S-benzylcysteine residues/mol of thyroglobulin (see above). Here again, S-benzylcysteine residues can be derived only from dehydroalanine residues.

**DISCUSSION**

When 2 iodotyrosine residues in thyroglobulin undergo enzymatic coupling which results in the formation of 1 hormone residue, the alanine side chain of 1 of the 2 precursor tyrosine residues is eliminated. We reported recently (7) that this "lost side chain" (2) appears in the enzymatic hydrolysate of thyroglobulin as pyruvic acid. The nature of the lost side chain in thyroglobulin before its hydrolysis remained unknown.

The calculation of these molar ratios is based on the assumption that the specific activities of all carbon atoms of [U-14C]tyrosine are identical. Although there might be some deviations from the mean specific activity of all carbon atoms, such deviations are insignificant according to the manufacturer (Amersham). Probably they do not exceed about 5%. See also J. C. Turner (13).
We could now show that the pyruvic acid is derived from dehydroalanine residues. We have proven this by two independent methods: 1) addition of benzyl mercaptan to the double bond of the dehydroalanine residues which results in the formation of S-benzylcysteine residues, and 2) reduction of the dehydroalanine residues to alanine residues with sodium borohydride.2

The second method requires labeled dehydroalanine residues because thyroglobulin contains 400 to 500 alanine residues/mol (24-26). In iodinated [U-14C]tyrosine-labeled thyroglobulin which we used for the reduction with sodium borohydride, the dehydroalanine residues are labeled because they are derived from labeled tyrosine residues.

In the first method, it is not necessary to use labeled thyroglobulin since the addition product, S-benzylcysteine, is not present in thyroglobulin. However, a large amount of thyroglobulin hydrolysate (see "Results") must be applied to the resin column of the amino acid analyzer well separated from other amino acids. The captoethanol, or 2-mercaptoethanesulfonic acid, because the addition product, S-benzylcysteine, is a commercially available compound and emerges from the long column of an amino acid analyzer well separated from other amino acids. The thyroglobulin used in these experiments contained ~60 atoms of iodine/mol and 2.9 hormone residues/mol. Reaction with benzyl mercaptan led to the formation of 2.5 to 3.0 S-benzylcysteine/hormone molar ratio of 0.9 to 1.0. As expected, S-benzylcysteine was not present in the control hydrolysate obtained from a thyroglobulin which had not been exposed to benzyl mercaptan.

Further proof for the formation of 1 dehydroalanine residue for each hormone residue synthesized in thyroglobulin in the course of its iodination was obtained by the reduction of [U-14C]tyrosine-labeled thyroglobulin with sodium borohydride (Method 2). [14C]Alanine was present and [14C]acetic acid virtually absent in the hydrolysate after treatment with sodium borohydride, while [14C]alanine was absent and [14C]acetic acid present without such treatment (Fig. 2B). For each mole of [14C]acetic acid formed in the control experiment, 1 mol of [14C]alanine was formed after reduction with sodium borohydride. Thus, both labeled compounds are derived from a common precursor, dehydroalanine residues.

Furthermore, each molecule of [14C]acetic acid and of [14C]alanine present in the acid hydrolysates corresponds to 1 molecule of [14C]tyrosine (Fig. 2B). Labeled acetic acid and labeled alanine are derived from labeled dehydroalanine residues and labeled tyronine is derived from labeled hormone residues.

In our earlier experiments (7) in which enzymatic hydrolysis of thyroglobulin was used instead of acid hydrolysis, labeled pyruvic acid was found instead of labeled acetic acid. The latter has been formed from the former by decarboxylation during the heating with acid. In the earlier experiments, the pyruvic acid/hormone molar ratio also was 1. Hence, both labeled pyruvic acid and labeled acetic acid are derived from labeled dehydroalanine residues which, in turn, are formed concomitantly with hormone residues. The [14C]acetic acid/hormone and [14C]pyruvic acid/hormone molar ratio of 1, thus, show that dehydroalanine residues are the sole product of the lost side chain. The conversion of certain (hormonogenic) tyrosine residues in thyroglobulin to hormone residues and dehydroalanine residues and the identification of the dehydroalanine residues as S-benzylcysteine, pyruvic acid, acetic acid, and alanine after addition of benzyl mercaptan to the double bond, enzymatic hydrolysis, acid hydrolysis, and reduction of the double bond with sodium borohydride, respectively, are summarized in the following scheme (Fig. 3).

This scheme also shows the stoichiometric relationship between the hormone residues and dehydroalanine residues formed in the course of the iodination of thyroglobulin (molar ratio = 1).

![Fig. 3. Schematic presentation of thyroid hormone synthesis in thyroglobulin. The arrow line indicates the polypeptide chain of thyroglobulin. Compounds in square brackets are intermediates that have not been isolated.](image-url)

The mechanism of the conversion of the side chain of certain tyrosine residues to dehydroalanine residues remains obscure. Nearly 40 years ago, Johnson and Tewkesbury (3) proposed a mechanism involving the oxidation of iodotyrosine to an iodotyrosyl phenoxy radical, followed by coupling of two such radicals with formation of a quinol ether. According to this hypothetical mechanism, the quinol ether then undergoes fission with formation of hormone and dehydroalanine. Although our findings are compatible with this hypothesis, it is doubtful whether thyroid peroxidase-catalyzed coupling which takes place within the catalytic site of the enzyme actually follows such a course. The first step in this scheme is quite plausible since peroxidases promote 1-electron transfers (27). However, there is no experimental proof to support the subsequent steps.

Dehydroalanine residues have been found in various naturally occurring peptide antibiotics and toxins. (For a review see Ref. 28) The largest of these peptides, nisin (15) and subtilin (29), contain 34 and 32 amino acid residues, respectively. The presence of dehydroalanine residues in histidine ammonia lyase and in phenylalanine ammonia lyase is still controversial (see Footnote 4 of Ref. 23). A study of the biogenesis of dehydroalanine residues in bacterial peptides would be of interest. These residues may have been formed from serine or cysteine residues. Conversion of [14C]serine to dehydroalanine residues of Bernaminycin has been reported

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2 Both methods have been used for the detection of dehydroalanine residues in peptides and proteins. The addition of mercaptans to dehydroalanine and 2,3-dehydrobutyrin (2-amino crotonic acid) residues in peptide antibiotics has been investigated by Gross and co-workers (14-17) and the reduction of these residues in other peptide antibiotics with sodium borohydride by Muramatsu and co-workers (18, 19) and by Bodansky et al. (20). Reduction with sodium borohydride has also been used in order to determine whether dehydroalanine residues are present in histidine ammonia lyase (21-23).
Formation of Dehydroalanine during Thyroid Hormone Synthesis

recently (30). In thyroglobulin where the formation of dehydroalanine residues is closely linked with that of thyroid hormone residues, the mechanism of the biosynthesis of dehydroalanine residues appears to be quite different.

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