Enzymatic Mechanism of Thyroxine Biosynthesis. Identification of the "Lost Three-Carbon Fragment"

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Although the biosynthesis of thyroid hormones has been investigated for over 50 years, the molecular mechanism of this important process is still not fully understood.[1] It is generally believed that the formation of thyroxine (T_4) proceeds via the oxidative free radical coupling of two 3,5-diiodotyrosine (DIT) units to generate T_4.[2] Two mechanisms were proposed for this oxidative phenolic coupling process.[3] The intramolecular mechanism entails the coupling of two properly juxtaposed DIT residues in the thyroglobulin molecule to form a T_4 residue in the polypeptide backbone of thyroglobulin; subsequent proteolysis of thyroglobulin generates free T_4.[1,2] The intermolecular mechanism is based on the observation that both free DIT and bound DIT residues in thyroglobulin readily coupled with 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA), derived from DIT via enzymatic transamination, to form T_4 under oxidative conditions.[4] Although it is the general notion that T_4 is biosynthesized primarily via intramolecular coupling,[1] it remains unclear as to whether T_4 formation in vivo operates via the intramolecular or intermolecular coupling mechanism or both.

As early as 1939, Von Mutzenbecher[5a] first reported that free DIT molecules could be converted into T_4 chemically, a result that was subsequently confirmed by Johnson and Tewkesbury[5b] in 1942. Since then, much effort has been devoted to defining the so-called "lost C3 fragment" that is formed when two molecules of DIT couple to form one molecule of T_4. The "lost C3 fragment" was characterized by different laboratories as dehydroalanine,[6a] hydroxypyruvic acid,[6b] alanine,[6c] serine,[6d] and pyruvic acid.[5b]

However, based on the extensive studies by Cahmann and coworkers[7] over a 10 year period, most investigators currently favor dehydroalanine as the "lost C3-fragment". In these studies the putative dehydroalanine residue in thyroglobulin was first reduced with sodium borohydride or trapped with benzyl mercaptan. After hydrolysis, the resulting products were characterized as [^{14}C]-alanine and benzylcysteine by a comparison of their retention times using ion-exchange column chromatography.[7] These results formed the cornerstone for the currently accepted mechanism of thyroid hormone biosynthesis.[1a,4a] Inherent in this mechanism is the intramolecular coupling of two DIT residues to generate a quinol ether intermediate, **3**, which rearranges to produce a dehydroalanine residue, **4** (Scheme 1). As no direct spectroscopic analyses of the products were made due to the extremely low yield of products formed, this structural assignment of the "lost C3-fragment" is not compelling. Consequently, the biosynthetic mechanism of T_4 formation warrants further study. We herein report our results showing that the "lost C3 fragment" is aminomalonic semialdehyde and not dehydroalanine as is claimed.

Because only 2 to 3 mol of T_4 are formed from 1 mol of thyroglobulin (M_r 660000),[8] it would be difficult to isolate sufficient quantities of products for characterization using spectroscopic methods. Hence we decided to use the nonapeptide **5**, K-DIT-K_5-DIT-K[9] as the substrate for our studies for it gives higher yields of T_4, so that the products could be characterized more definitively. Since this oxidative coupling could be catalyzed by lactoperoxidase (LPO), myeloperoxidase (MPO), and horseradish peroxidase[10] (HRP), we used LPO for our initial studies. As the product profile varied markedly with reaction conditions, it was necessary to optimize the incubation conditions. After much experimentation, a suitable reaction condition was found to be as follows: To 1 mg (0.57 μmol) of the nonapeptide, **5**, was added LPO (10.4 units), glucose oxidase (2.8 units), and glucose (5 μmol) in 1 mL of 50 mM sodium phosphate buffer, pH 7.4. After incubation for 30 min at 37 °C, the HPLC profile of the mixture

Table 1. LPO and TPO-Catalyzed Oxidative Coupling of **5**

$$\text{K-DIT-K}_5\text{-DIT-K} \xrightarrow[\text{pH 7.4, 37 °C}]{\text{peroxidase/[H}_2\text{O}_2]} \text{products}$$

products	HPLC[a]			ESIMS (LC-MS)		
	time (min)	yield (%) LPO[b]	yield (%) TPO[c]	M_r calcd	found LPO	found TPO
K-DIT-K_5-DIT-K	13.5	72	84	1745.2	1744.5	1744.8
K-T_4-K_5-(Ald)G-K[d]	20.8	2.0	0.7	1759.2	1759.0	1759.1
K-*cyclo*[T_3-K_5-MIT]-K[e]	23.1	0.8	0.1	1835.2	1834.8	1835.3
K-T_4-K_5	29.3	1.7	0.2	1545.9	1545.7	1546.1
K-T_4-K_5-DIT-K[f]	34.2		1.1	2089.1		2088.5
K-T_4-K_5-(acid)G-K[g]	34.9	1.0	0.6	1775.2	1774.9	1775.1
K-DIT-K_5-T_4-K[f]	37.1	8.5	0.4	2089.1	2088.5	2088.8
K-T_4-K_5-T_4-K	38.0	0.8		2433.0	2432.2	

[a] HPLC analyses were performed on a C_{18} cartridge (8 × 100 mm) with an elution gradient from 10% to 22% acetonitrile with 0.1% TFA over 30 min, then from 22% to 63% over 20 min, at a flow rate of 1.5 mL/min. [b] LPO: **5** (1 mg, 0.57 μmol) was incubated with LPO (10.4 units), D-glucose (5 μmol), and glucose oxidase (2.8 units) in a total volume of 1 mL of sodium phosphate buffer (pH 7.4) for 30 min. [c] TPO: **5** (0.05 mg, 0.029 μmol) was incubated with TPO (3.72 units), D-glucose (0.25 μmol), and glucose oxidase (0.14 units) in a total volume of 0.6 mL of sodium phosphate buffer (pH 7.4) for 30 min. [d] *Or* K-(Ald)-G-K_5-T_4-K. [e] Product of intramolecular ortho C−C coupling. [f] May be interchangeable. [g] *Or* K-(acid)G-K_5-T_4-K.

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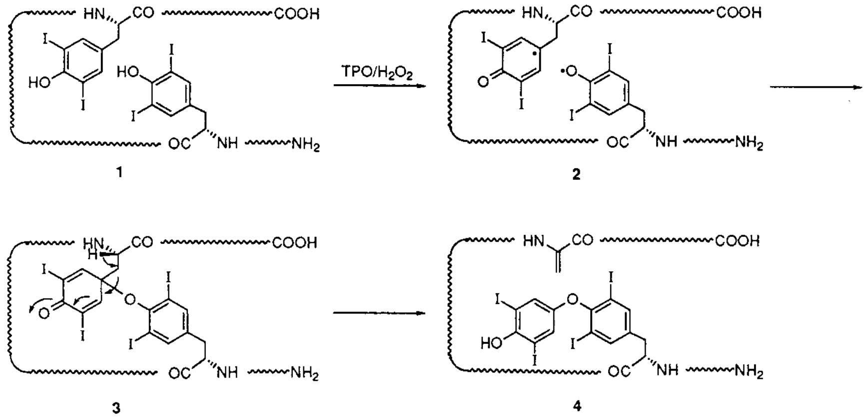
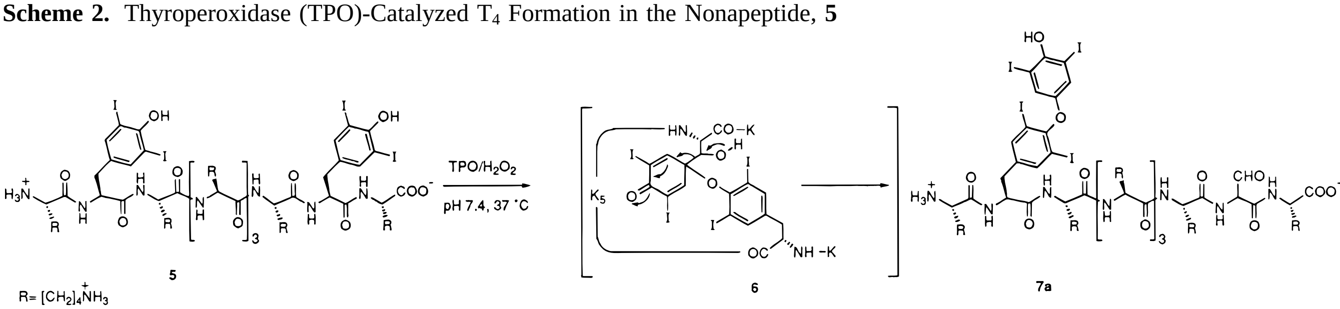
Scheme 1. The Dehydroalanine Mechanism for T_4 Formation in Thyroglobulin



Scheme 2. Thyroperoxidase (TPO)-Catalyzed T_4 Formation in the Nonapeptide, **5**



showed the presence of several prominent peaks, which were analyzed using LC-ESIMS (Table 1). In all the experiments, a peak with a retention time of 20.8 min was observed. Its mass of 1759 is consistent with the structure of an aldehyde intermediate, K-T_4-K_5-(Ald)G-K, **7a**, or K-(Ald)G-K_5-T_4-K, **7b** (M_r 1759) [(Ald)G = malonic semialdehyde], formed via intramolecular coupling of two DIT residues to afford a T_4 residue and the aldehydic C3-fragment in the same peptide. Mass 1545 corresponds to K-T_4-K_5, originated from the hydrolysis of **7a** by the loss of (Ald)G-K. The presence of the peak with a mass of 1775, corresponding to K-T_4-K_5-(acid)G-K (M_r 1775), further supports this structural assignment. Mass 2089 may be assigned to a product derived from intermolecular coupling, where the DIT residue in one molecule of **5** coupled with the DIT residue in another molecule of **5** to give the T_4 peptide, K-T_4-K_5-DIT-K (M_r 2089), and the corresponding aldehydic counterpart, K-DIT-K_5-(Ald)G-K (M_r 1415). It is noteworthy that the former molecule (M_r 2089) underwent another intermolecular coupling to yield the product K-T_4-K_5-T_4-K (M_r 2432).

To confirm the structural assignment of the aldehydic peptide **7**, another nanopeptide, A-DIT-K_4-A-DIT-A, was synthesized and incubated with LPO under similar reaction conditions. LC-ESIMS analysis of the incubation mixture showed two peaks with the same mass (1588), which coincides with the structures of the expected aldehydic peptides, A-T_4-K_4-A-(Ald)G-A and A-(Ald)G-K_4-A-T_4-A. This result indicates that the intramolecular coupling process lacked regioselectivity.

The possibility that **7a** or **7b** could be derived from an analogous dehydroalanyl peptide intermediate, **4**, via the addition of water followed by peroxidase-catalyzed oxidation of the serinyl peptide was ruled out by two separate control experiments. First the K-T_4-K_5-S-K peptide was synthesized and then was exposed to LPO under the reaction conditions used when **5** was converted into **7**. However, careful mass spectral analyses of LC/MS did not reveal any compound with a mass corresponding to **7**, indicating that the primary alcohol was not oxidized under these conditions. Moreover, neither K-SBC-K_5-T_4-K nor K-T_4-K_5-SBC-K (SBC = *S*-benzylcysteinyl) was detectable by LC/MS analysis of the reaction mixture, which was obtained from incubating **5** with LPO under the same aforementioned conditions, followed by treatment of the resulting mixture with benzylmercaptan.

Having defined the optimum reaction conditions for oxidative coupling, we next turned our attention to incubations using partially purified TPO.[11] Using the same reaction conditions, the pattern of oxidative coupling for TPO was found to be virtually the same as that of LPO (Table 1). Again prominent peaks for the intramolecular coupling product, **7** (**7a** or **7b**), as well as the intermolecular product (K-T_4-K_5-DIT-K) were observed.

In conclusion, we have characterized the "lost C3-fragment", generated during the enzymatic oxidative coupling of the peptide, **5** (K-DIT-K_5-DIT-K), to form thyroxine (T_4). Our studies provide the first direct evidence to clearly show that the "lost C3-fragment" is not a dehydroalanine residue as is claimed, but rather it is an aminomalonic semialdehyde residue, embedded in the peptide backbone. The intramolecular coupling product, **7** (K-T_4-K_5-(Ald)G-K or K-(Ald)G-K_5-T_4-K), was identified using ESIMS. Further, our results showed that both intra- and intermolecular mechanisms could operate in the biosynthesis of thyroid hormones. Our results strongly implicate the intermediacy of a hydroxy quinol ether, **6**, which facilitates C−C bond scission via reverse aldolization to form a peptide containing T_4 and the C3 aldehyde as shown (Scheme 2). This mode of carbon−carbon bond cleavage is in accord with results of our previous model studies.[4a] Further, benzylic oxidation of dihalotyrosine derivatives by horseradish peroxidase has been previously reported.[12] While the precise mechanism for the formation of the aldehydic peptide, **7**, is not yet established, our findings rule out the currently accepted dehydroalanine mechanism of thyroxine formation (Scheme 1). The intimate details of thyroid hormone biosynthesis are currently under investigation and the results will be reported at a later date.

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Supporting Information Available: Experimental procedures and characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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