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Mechanism of thyroid hormone biosynthesis. Enzymatic oxidative coupling of 3,5-diiodo-L-tyrosine derivatives

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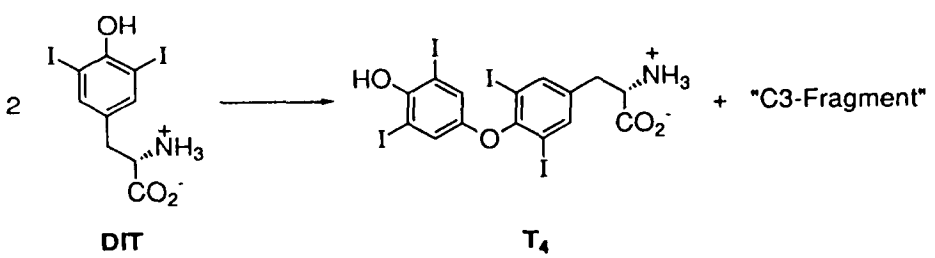
Abstract

Both enzymatic and non-enzymatic oxidative coupling of 3,5-diiodo-L-tyrosine derivatives afforded a T_4 derivative and a C_3 fragment. The C_3 fragment is not dehydroalanine as was claimed by previous workers but rather it was identified as aminomalonic semialdehyde, which decomposed to hydroxypyruvate, **3**, in the acidic work-up. The latter was isolated as its osazone derivative, **6**. © 1999 Published by Elsevier Science Ltd. All rights reserved.

The biosynthesis of thyroxine (T_4) has been investigated for over 50 years, but the mechanistic details of this important process remain poorly understood.[1] It is generally believed that T_4 is derived from the oxidative free radical coupling of two 3,5-diiodo-L-tyrosine (DIT).[2] Two mechanisms have been proposed for the biosynthesis of T_4 in the thyroid gland.[3] The intramolecular mechanism involves the coupling of two residues of DIT within the thyroglobulin (TGB) molecule to yield T_4 and a three carbon unit.[1,2] Intermolecular coupling is based on the observation that under suitable oxidizing conditions, DIT coupled very readily with its keto analog, 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) to form T_4 (Scheme 1).[4] Moreover, DIHPPA also reacted readily with DIT residues in TGB to form T_4.[4c] Recently, we have shown that DIHPPA was first converted into highly reactive quinol epoxy intermediates,[4a,b] which then coupled with DIT to generate T_4. However, at the present time, it is unclear whether T_4 formation in vivo proceeds via the intramolecular or the intermolecular mechanism or both.



Scheme 1.

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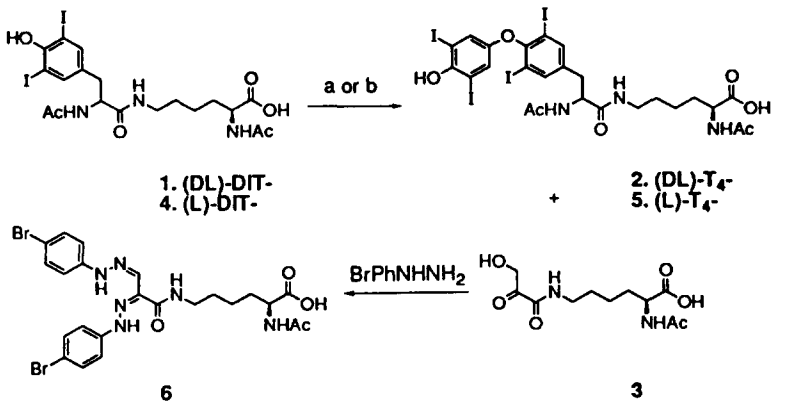
As early as 1939, Von Mutzenbecher first reported that free DIT molecules could be converted into T_4 non-enzymatically.[5] Since that time, much work has been devoted to the characterization of the so called 'lost C_3 fragment' because its identification could provide valuable insight into the mechanism of this oxidative coupling reaction. This 'lost C_3 fragment', generated when a donor DIT molecule is transferred to an acceptor DIT residue, was identified by different laboratories as dehydroalanine, alanine, hydroxypyruvic acid, serine and pyruvic acid.[6] However, extensive studies conducted by Cahmann and co-workers[7] have claimed that this C_3 fragment is dehydroalanine, which served as the cornerstone in the formulation of the currently accepted mechanism of T_4 biosynthesis.[1a,2,3a]

Our continuing interest in defining the mechanism of T_4 biosynthesis led us to examine the enzymatic intermolecular oxidative coupling of DIT derivatives with special reference to the characterization of the 'lost C_3 fragment.' In this communication, we report our experimental results verifying that the C_3 fragment is aminomalonic semialdehyde and not dehydroalanine as was reported.[7,8] Since it was reported that air oxidation of free DIT afforded T_4 albeit in low yields, we decided to reexamine this coupling reaction with a view to isolating and characterizing the three carbon fragment. For this purpose, we subjected *N*-acetyl-L-DIT to air oxidation and obtained 3.1% of *N*-acetyl-T_4 and 0.8% of hydroxypyruvic acid, isolated as its osazone derivative. We then turned our attention to the enzymatic coupling of *N*-acetyl-L-DIT. It is well known that both horseradish peroxidase and lactoperoxidase are capable of catalyzing this oxidative phenolic coupling to form T_4.[9] However, we have previously noted that the predominant product of HRP oxidation of *N*-acetyl-L-DIT is *N*-acetyl-dityrosine, a product of C–C coupling.[10] In fact, upon reexamination, only a small quantity of *N*-acetyl-T_4 was detected but the yield was too low for more rigorous characterization of the products.

In 1958, Pitt-Rivers and James[6c] reported that air oxidation of *N*-acetyl-DL-DIT-ε-*N*-(α-*N*-acetyl)-L-lysine, **1**, afforded the corresponding T_4 derivative, **2**, in 40–50% yield, accompanied by hydroxypyruvic acid, **3**, isolated as its hydrazone derivative. Both **2** and the hydrazone of **3** were characterized by C, H, N microanalyses and melting point determinations. Because of the high yield of products obtained in this non-enzymatic oxidation, we prepared *N*-acetyl-L-DIT-ε-*N*-(α-*N*-acetyl)-L-lysine, **4**, as a model substrate for the C_3 fragment determination experiment. Thus, compound **4** (258 mg) was dissolved in 2 ml of 0.2 M sodium borate buffer, pH 7.5, at 38°C and left standing for 11 days. The progress of the reaction was monitored by HPLC. The reaction was terminated by the addition of $NaHSO_3$ and excess $BrPhNHNH_2$ was added to trap the C_3 fragment. Two products were isolated which were characterized as the T_4 derivative **5** (16.2%) and the osazone **6** (20%) on the basis of their spectroscopic data.[11] These results are in agreement with the structural assignments proposed by Pitt-Rivers and James.[6c]

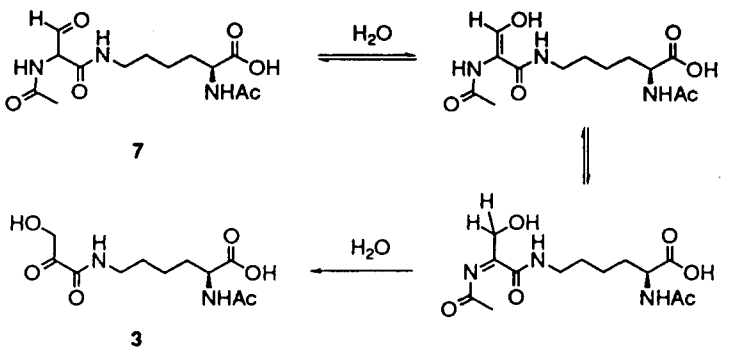
In contrast to air oxidation of **4**, the obtainable yields in the enzymatic oxidation of **4** varied in the range of only 0.4 to 1.7% (HPLC). Higher yields of **5** were obtained by the addition of a stoichiometric amounts of DIT to the incubation mixture, an observation that is consistent with published reports that DIT enhanced the formation of T_4.[9a,12] After much experimentation, the following reaction conditions were found to be optimal for preparative purposes: a mixture consisting 1 mmol each of **4** and DIT were incubated with 80 000 units of horseradish peroxidase (HRP) and a H_2O_2 generating system (1.08 g of glucose and 3000 units of glucose oxidase) in 50 ml of 0.2 M Tris–HCl buffer, pH 7.4, at 37°C. After 2 h, the reaction was quenched with $NaHSO_3$ and the C_3 fragment was trapped with an excess amount of $BrPhNHNH_2$. The product mixture was subjected to HPLC purification to yield the T_4 derivative, **5**, and the C_3 bearing fragment, **6**, in 0.6 and 1.2% yields, respectively (Scheme 2). These results indicated that the enzymatic oxidative coupling of DIT derivatives proceeded via intermolecular coupling to give the same products as those obtained from air oxidation of **4**.

However, it is most likely that **6** was derived from an unstable aldehydic derivative, **7**, that underwent hydrolysis during work-up to yield the hydroxypyruvate derivative, **3**, which upon reaction with an excess



Scheme 2. (a) Air, pH 7.5, 38°C, 11 days; (b) HRP/H_2O_2, pH 7.4, 37°C, 2 h

of bromophenylhydrazine gave the osazone derivative **6** (Scheme 3). This proposal is in agreement with results in our model studies.



Scheme 3.

In conclusion, we have trapped and isolated a hydroxypyruvic acid derivative **6** during the enzymatic oxidative coupling of **4** to form thyroxine, T_4. Both air oxidation and enzymatic oxidative coupling afforded the same two products. These derivatives were characterized using ^1H and ^{13}C NMR as well as HR MALDI MS. Our results showed that the 'lost C_3 fragment' is not dehydroalanine as was claimed by many workers but it is *N*-acetyl aminomalonic semialdehyde, which was hydrolyzed to hydroxypyruvic acid in the acidic work-up conditions. While the exact mechanism for the formation of the aldehydic intermediate is still not clear, it is likely that this oxidative coupling may involve a hydroxy quinol ether, which can readily generate the C_3-fragment via non-enzymatic reverse aldolization. The precise mechanism of thyroxine mechanism is being studied and these results will be reported later.

Acknowledgements

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11. *N*-Acetyl-L-thyroxyl-ε-*N*-(α-*N*-acetyl)-L-lysine, **5** (32 mg, 16.2%): ^{1}H NMR (methanol-d_4) 7.75 (s, 2H), 7.00 (s, 2H), 4.43 (t, *J*=7.5, 1H), 4.28 (dd, *J*=5.0, 1H), 3.07 (m, 2H), 2.95 (dd, *J*=7.4, 1H), 2.79 (dd, *J*=8.0, 1H), 1.92 (s, 3H), 1.89 (s, 3H), 1.77 (m, 1H), 1.64 (m, 1H), 1.39 (m, 2H), 1.32 (m, 2H); ^{13}C NMR (methanol-d_4) 175.5, 173.4, 173.0, 172.7, 153.8, 152.4, 151.7, 142.4 (2C), 140.1, 127.1 (2C), 91.5 (2C), 85.7 (2C), 56.0, 53.6, 40.1, 37.6, 32.3, 30.1, 24.2, 22.4, 22.3; HR MALDI MS calcd for $C_{25}H_{26}I_4N_3O_7$ ([M–H]$^-$) 987.795, found 987.796. Osazone **6** (25 mg, 20%): ^{1}H NMR (methanol-d_4) 8.14 (s, 1H), 7.41 (d, *J*=8.8, 2H), 7.37 (d, *J*=9.0, 2H), 7.24 (d, *J*=9.0, 2H), 6.86 (*J*=8.8, 2H), 4.30 (dd, *J*=4.8, 1H), 3.30 (d, *J*=6.6, 2H), 1.90 (s, 3H), 1.80 (m, 1H), 1.65 (m, 1H), 1.55 (m, 2H), 1.41 (m, 2H); ^{13}C NMR (methanol-d_4) 175.2, 172.7, 166.2, 144.4, 143.7, 134.0, 133.5 (4C), 129.0, 117.4 (2C), 115.5, 115.3 (2C), 113.1, 53.5, 32.3, 30.7, 30.4, 24.3, 22.7; HR MALDI MS calcd for $C_{23}H_{27}Br_2N_6O_4$ ([M+H]$^+$) 609.046, found 609.046; calcd for $C_{23}H_{25}Br_2N_6O_4$ ([M–H]$^-$) 607.030, found 607.037.
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