Model Reactions of Thyroxine Biosynthesis. Identification of the Key Intermediates in Thyroxine Formation from 3,5-Diiodo-L-tyrosine and 4-Hydroxy-3,5-diiodophenylpyruvic Acid

Vibha B. Oza, Grzegorz M. Salamonczyk, Zhi-wei Guo, and Charles J. Sih*

School of Pharmacy, University of Wisconsin 425 North Charter Street, Madison, Wisconsin 53706-1515

Received June 10, 1997

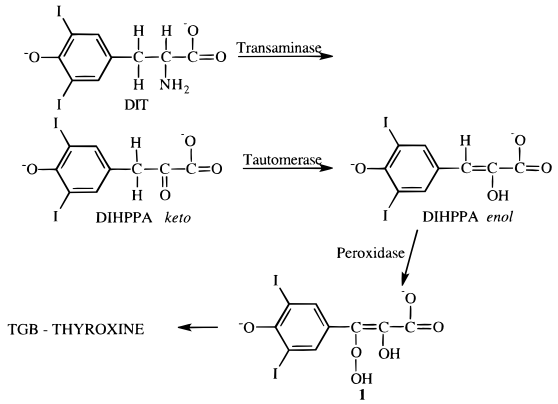
Although it was suggested 50 years ago that thyroxine (T_4) is formed in the thyroid from its precursor 3,5-diiodo-L-tyrosine (DIT),[1] the mechanism of this conversion is still not completely understood. Two possible mechanisms have been proposed for the formation of T_4 in the thyroid gland, and these are generally referred to as intramolecular and intermolecular coupling.[2]

Intramolecular coupling involves oxidation of DIT to a free radical and interaction of two DIT radicals to form T_4 through a quinol ether intermediate.[3] Earlier workers envisaged the coupling reaction to involve the conversion of free DIT to free T_4.[4] However, later studies indicated that peptide-linked DIT is more likely the precursor of T_4 and that coupling of two molecules of DIT occurs within the thyroglobulin (TGB) molecule[3] to yield T_4 and dehydroalanine as the lost three carbon unit.[5]

Intermolecular coupling is based on the discovery that DIT couples very readily with its keto analog 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) to form T_4.[6] This reaction has been studied extensively by Cahnmann and co-workers[7] who showed that under suitable oxidizing conditions DIHPPA couples readily with DIT residues in TGB to form T_4.[8] They isolated an active intermediate in this reaction and proposed that its structure is the hydroperoxide of DIHPPA, **1**.[7b] A possible scheme for T_4 formation in the thyroid by intermolecular coupling was presented by Blasi et al.,[9] who postulated that DIHPPA may be formed from DIT in a reaction catalyzed by tyrosine transaminase (Scheme 1).[10] A tautomerase present in the thyroid and in other tissues then converts the DIHPPA to the enol form.[11] The latter is presumably oxidized by H_2O_2 and thyroid peroxidase[9] (TPO) to the putative hydroperoxide **1**, which was shown by Cahnmann and co-workers to be the intermediate that couples spontaneously with DIT.[12] Hence, there are cogent reasons to believe that the coupling reaction between DIHPPA and DIT may represent a biosynthetic model for T_4 formation in the thyroid.

However, at the present time it is not definitely known whether T_4 formation in vivo involves predominantly the intramolecular or the intermolecular coupling mechanism.[13] We decided to reexamine the chemical identity of the putative hydroperoxide intermediate **1** because it was difficult for us to rationalize on mechanistic grounds the unusually facile reactivity of this intermediate with DIT to form T_4. In this paper, we report the experimental data used in establishing the structures of the T_4 precursors as the epoxides **8a** and **9**.

Scheme 1. Blasi's Intermolecular Mechanism of Thyroxine Formation



When DIHPPA was subjected to the oxygenation conditions (0.2 M borate buffer, pH 7.5) as described by Cahnmann and co-workers,[7b] a complex mixture of products was formed. The reverse-phase HPLC profile of the reaction mixture showed the presence of six peaks.[14] Owing to the instability of the isolated compounds, they were kept at −78 °C and analyzed immediately. It was found that both peaks 3 and 5 decomposed completely to 4-hydroxy-3,5-diiodobenzaldehyde (DIHBA) and 3,5-diiodo-1,4-benzoquinone (DIBQ) within 24 h at 24 °C, whereas the other three peaks decomposed less readily only to DIHBA. Further, only peaks 3 and 5 reacted readily with DIT to form T_4.

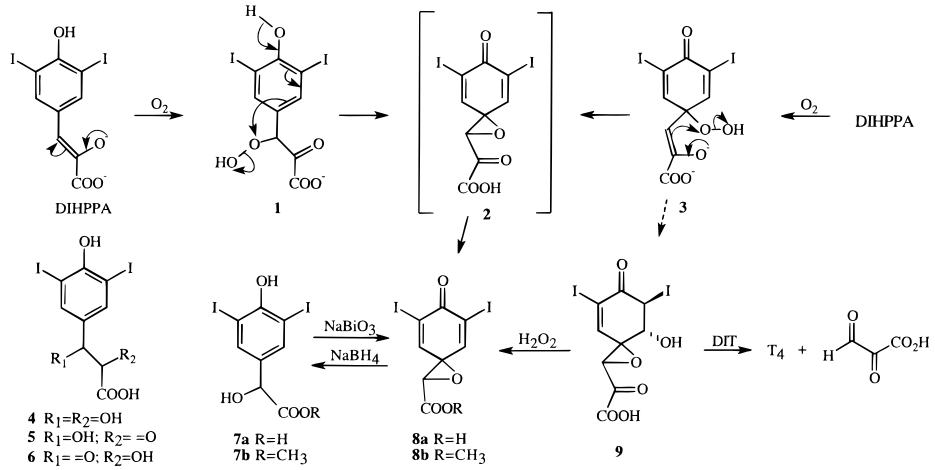
The major product, peak 5, was found to react readily with DIT to give T_4 in over 85% yield after 3 h of stirring at 24 °C. It was characterized as the quinol epoxide **8a** on the basis of its NMR spectral data and its reduction by sodium borohydride to furnish 4-hydroxy-3,5-diiodomandelic acid[15] (**7a**). Its identity was confirmed by the reaction of **7b** with an excess of sodium bismuthate[16] in an ethyl acetate/acetic acid/water solution to give **8b**, whose spectral properties and HPLC retention time were identical to those from a sample of **8b** obtained by reaction of **8a** with diazomethane.

The compound in peak 3 (proposed to be **9**) reacted with DIT even faster than **8a** to yield T_4 cleanly and quantitatively at 0 °C in less than 45 min. The other product of the reaction, mesoxalic acid semialdehyde, was trapped with phenylhydrazine to furnish an adduct whose physical properties coincided with those of the bis-diphenylhydrazone. Its retention time and mass spectral data were identical to an authentic sample prepared by the procedure of Fenton and Ryffel.[17] The presence of a keto

(1) Harington, C. R.; Barger, G. *Biochem. J.* **1927**, *21*, 169−183.
(2) Degroot, L. J.; Niepomniszcze, H. *Prog. Endocrinol. Met.* **1977**, 665−718.
(3) Taurog, A.; Dorris, M.; Doerge, D. R. *Arch. Biochem., Biophys.* **1994**, *315*, 82−89. See also: Johnson, T.; Tewkesbury, L. *Proc. Natl. Acad. Sci. U.S.A.* **1942**, *23*, 73−77.
(4) Harington, C. R.; Randall, S. S. *Biochem. J.* **1929**, *23*, 373−383.
(5) (a) Gavaret, J. M.; Nunez, J.; Cahnmann, H. J. *J. Biol. Chem.* **1980**, *255*, 5281−5285. (b) Gavaret, J. M.; Cahnmann, H. J.; Nunez, J. *J. Biol. Chem.* **1979**, *254*, 11218−11222.
(6) (a) Hillmann, G.; Keil, B.; Tashimi, P. *Z. Naturforsch.* **1961**, *16*, 28−32. (b) Meltzer, R. I.; Stanaback, R. J. *J. Org. Chem.* **1961**, *26*, 1977−1979.
(7) (a) Nishinaga, A.; Cahnmann, H. J.; Kon, H.; Matsuura, T. *Biochemistry* **1968**, *7*, 388−397. (b) Cahnmann, H. J.; Funakoshi, K. *Biochemistry* **1970**, *9*, 90−98.
(8) Toi, K.; Salvatore, G.; Cahnmann, H. J. *Biochim. Biophys. Acta* **1965**, *97*, 523−531.
(9) Blasi, F.; Fragomele, F.; Covelli, I. *Endocrinology* **1969**, *85*, 542−551.
(10) Igo, R. P.; Mahoney, C. P.; Limbeck, G. A. *Biochim. Biophys. Acta* **1968**, *151*, 88−98.
(11) Blasi, F.; Fragomele, F.; Covelli, I. *J. Biol. Chem.* **1969**, *244*, 4864−4870.
(12) Ogawara, H.; Cahnmann, H. J. *Biochim. Biophys. Acta* **1972**, *257*, 328−338.
(13) Taurog, A. In Endocrinology; DeGroot, L. J., Cahill, G. F., Jr., Martini, L., Nelson, D. H., Odell, W. D., Potts, J. T., Jr., Steinberger, E., Winegrad, A. I., Eds.; Grune and Stratton: New York, 1979; Vol. 1, pp 331−342.
(14) The oxygenated DIHPPA mixture was separated on a reverse-phase C_{18} column (19 × 300 mm) using a gradient elution system consisting of 80% of solvent A (0.1% in water) to 76% of solvent B (0.1% TFA in 90% aqueous acetonitrile) in 25 min. A flow rate of 8 mL/min was used. Retention times: peak 1, 11.8 min; peak 2, 15.3 min; peak 3, 16.3 min; peak 4, 17.3 min; peak 5, 20.8 min; peak 6, 24.3 min.
(15) Matsuura, T.; Cahnmann, H. J. *J. Am. Chem. Soc.* **1959**, *81*, 871−878.
(16) Adler, E.; Holmberg, K.; Ryrfors, L. O. *Acta Chem. Scand.* **1974**, *B28*, 883−887.
(17) Fenton, H. J. H.; Ryffel, J. H. *J. Chem. Soc.* **1902**, *81*, 426−434.

S0002-7863(97)01903-3 CCC: $14.00

Scheme 2. Products Derived from the Oxygenation of DIHPPA

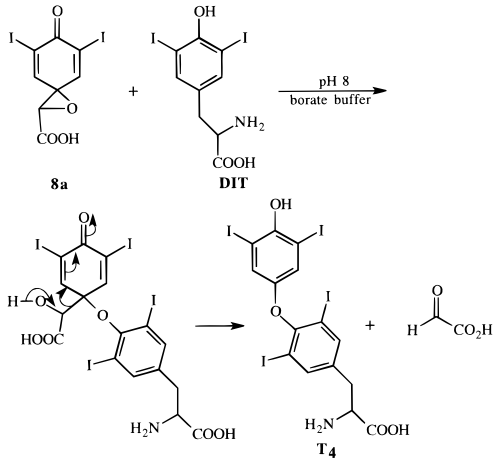


function in the side chain of **9** was confirmed by reduction of compound **9** with $NaBH_4$ to give 3-(4-hydroxy-3,5-diiodophenyl)-2,3-dihydroxypropionic acid (**4**). Further treatment of **9** with H_2O_2 afforded **8a**, whose methyl ester was identical to an authentic sample of **8b**. Although these experimental data suggested that the structure of the compound in peak 3 could be the quinol keto epoxide **2**, the ^1H and ^{13}C NMR spectra of this compound were more consistent with **9**,[18] a product derived from the 1,4-addition of OH to **2**.[19]

One could visualize two possible pathways for the oxygenation of DIHPPA, as illustrated in Scheme 2. Oxygenation occurs at the ipso position of DIHPPA to give **3**, which in turn serves as precursor to compounds **9** and **2**. Alternatively, DIHPPA undergoes hydroperoxygenation at the benzylic position to yield **1**, which gives rise to **2** as shown. However, the detailed mechanism for the formation of **9** from **2** and/or **3** requires further investigation. The oxirane **8a** could originate from both **9** and **2**, but it is unclear at what stage the decarboxylation reaction took place to form **8a**. The possibility of decarboxylation prior to oxygenation seemed remote, for the presence of the keto functionality at C-2 of the propionic acid side chain of DIHPPA is crucial for oxygenation to occur. This is supported by the observation that under the same oxygenation conditions 4-hydroxy-3,5-diiodophenylacetic acid or DIT remained unchanged even after 1.5 h. Also, when the phenolic hydroxyl of DIHPPA was acetylated, no oxygenation was noted. To gain further insights into the origin of **8a** and **9**, it was important to characterize the minor HPLC peaks.

Peak 1 consisted of a mixture of two compounds by NMR analysis, and many attempts to separate the mixture by HPLC were unsuccessful. The NMR data were consistent with the proposal that peak 1 was a mixture of 3-(4-hydroxy-3,5-diiodophenyl)-3-hydroxy-2-oxopropionic acid (**5**) and its isomer, 3-(4-hydroxy-3,5-diiodophenyl)-2-hydroxy-3-oxopropionic acid (**6**). This proposition was supported by the observation that $NaBH_4$ reduction of peak 1 afforded only **4**, the same diol as that obtained from reduction of **9**. Since NMR analysis of peak 1 after treatment with triphenylphosphine or sodium bisulfite showed no change, it is unlikely that the components of the mixture contained a hydroperoxy function. One could easily envisage that **6** originated from **5** via keto−enol tautomerism under the mildly basic incubation conditions. As expected, **5** and **6** did not react with DIT to form T_4. Mass spectral analysis of **5** and **6** afforded a principal fragment at *m*/*e* 404 corresponding to M − CO_2.

Scheme 3. T_4 Formation from the Reaction of DIT with **8a**



Peak 2 was found to be identical to an authentic sample of 4-hydroxy-3,5-diiodomandelic acid (**7a**) and as such did not react with DIT to give T_4. Its retention time and spectral data were identical to those of **7a** obtained from reduction of **8a**. Unfortunately, the quantity of peak 4 was too small for conclusive spectroscopic characterization, but this compound also did not react with DIT to form T_4. Peak 6 was identified as DIBQ.

In conclusion, we have isolated and rigorously characterized the reactive intermediates involved in thyroxine formation during the oxygenation of DIHPPA. Our study constitutes the first conclusive experimental proof that the intermediate is not 3-(4-hydroxy-3,5-diiodophenyl)-3-hydroperoxy-2-oxopropionic acid (**1**) as was claimed, but rather it is the epoxides **8a** and **9**. One could now explain the remarkable chemical reactivity of the putative T_4 precursors, for the electrophilic carbons of **8a** and **9** greatly facilitate SN2 attack by DIT to yield the respective unstable vinylogs of β-hydroxy ketones,[19] which in turn undergo spontaneous reverse aldolization to form T_4 and the respective lost carbon fragments (Scheme 3). The clean and quantitative reactions of **8a** and **9** with DIT at low to ambient temperatures at neutral pH strongly suggest the involvement of DIHPPA in thyroxine biosynthesis. The exact role of these reactive quinol epoxide intermediates **8a** and **9** in the overall mechanistic scheme of thyroxine biosynthesis is currently under investigation and will be reported at a later date.

Acknowledgment. This investigation was supported in part by a grant from the NIH. We thank Mr. Gary Girdaukas for his capable assistance with the low temperature NMR experiments.

Supporting Information Available: Experimental procedures and characterization data (4 pages). See any current masthead page for ordering and Internet access instructions.

JA9719034

(18) Compound 9: ^1H NMR (CD_3CN, 0 °C) δ 7.65 (s, 1H), 5.55 (d, J = 12.0 Hz, 1H), 4.95 (d, J = 12.0 Hz, 1H), 4.30 (s, 1H); ^{13}C NMR (CD_3CN, 0 °C) δ 185.1, 171.5, 168.1, 150.1, 101.8, 84.08, 65.91, 65.06, 35.64.

(19) Barton, D. H. R.; Deflorin, A. M.; Edwards, O. E. *J. Chem. Soc.* **1956**, 530−534. Battersby, A. R. *Proc. Chem. Soc.* **1963**, 189−500.

(20) Sih, C. J.; Lee, S. S.; Tsong, Y. Y.; Wang, K. C. *J. Biol. Chem.* **1966**, *241*, 540−550.