Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 512-517

BIOSYNTHESIS OF BIOPTERIN STUDIES ON THE MECHANISM OF 6-PYRUVOYLTETRAHYDROPTERIDINE SYNTHASE

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Received December 14, 1987

[1'-^{3}H]- and [2'-^{3}H]dihydroneopterin triphosphate (NH_2TP) were prepared enzymatically from [4-^{3}H]- and [5-^{3}H]glucose and converted to tetrahydrobiopterin (BH_4) by an extract from bovine adrenal medulla. The formation of BH_4 from both [1'-^{3}H]- and [2'-^{3}H]-NH_2TP proceeds with virtually complete loss of the respective tritium label. The breaking of the CH-bond at C-1' is characterized by a kinetic isotope effect of 2.6 $\pm$ 0.5. A smaller kinetic isotope effect of 1.5 $\pm$ 0.2 was found for the breaking of the CH-bond at C-2'.

The first committed step in the biosynthesis of tetrahydrobiopterin (BH_4) is the formation of dihydroneopterin triphosphate (NH_2TP) by ring expansion of GTP yielding NH_2TP (for review see Ref. 1) (Fig. 1). The subsequent steps have been controversial for many years. On the basis of recent work from several groups, it is now generally accepted that NH_2TP is converted to pyruvoyltetrahydropterin (PPH_4) by the enzyme, pyruvoyltetrahydropterin synthase (2-6). The enzyme has been purified to homogenity from human liver (7). The mechanism of this reaction is incompletely understood. This paper describes studies using [1'-^{3}H]-NH_2TP and [2'-^{3}H]-NH_2TP as substrates of PPH_4 synthase.

MATERIALS AND METHODS

GTP cyclohydrolase I (8), phosphoribosylpyrophosphate synthetase (9), and guanine phosphoribosyltransferase (10) were isolated from *Escherichia coli* by published procedures. Cell extract from bovine adrenal medulla was prepared and partially purified by ammonium sulfate precipitation and gel permeation chromatography according to published procedures (11).

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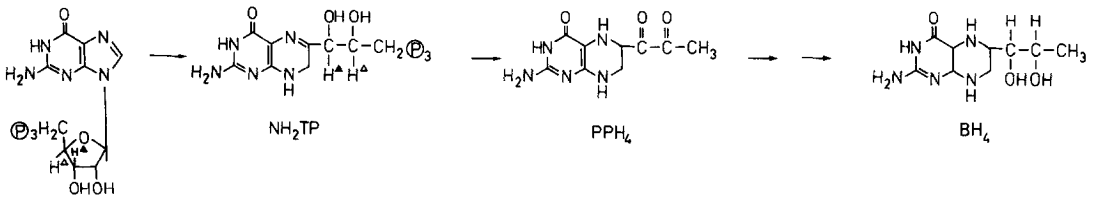

Fig. 1. Biosynthesis of biopterin

[3-^{3}H]- and [4-^{3}H]ribose 5-phosphate were prepared from [4-^{3}H]- and [5-^{3}H]-glucose, respectively (12). The ribose phosphates were converted to [3'-^{3}H]- and [4'-^{3}H]GMP, respectively, by the action of phosphoribosyl pyrophosphate synthetase and guanine phosphoribosyltransferase in analogy to a published procedure (13). GMP was purified by ion exchange HPLC and converted to GTP by the action of guanylate kinase and pyruvate kinase (14). GTP was again purified by ion exchange HPLC.

Enzyme assays contained 8 uM GTP (specific activity, 0.18 Ci/mmol), 1 mM $MgCl_2$, 50 mM Tris chloride pH 7.4, and 0.2 units of GTP cyclohydrolase in a volume of 127 ul. The mixture was incubated for 1 h to convert GTP to NH_2TP. NADPH (0.21 umol) and partially purified adrenal cell extract (4 ul) were added, and the mixture was incubated for appropriate intervals.

Neopterin triphosphate (NTP), neopterin, biopterin and pterin were isolated by reversed phase HPLC (Nucleosil 10 C_{18} column, 4 x 250 mm; eluent, 30 mM ammonium formate with 30 mM formic acid). Elution was monitored fluorometrically.

RESULTS

Fig. 2 shows two hypothetical reaction sequences for PPH_4 synthase (7). It is assumed that the reaction is initiated by the abstraction of a H^+ ion from either C-1' or C-2' of NH_2TP by a basic group of the enzyme. In a later reaction step, a H^+ ion is again abstracted from the neighbouring CH group. Depending on the solvent accessibility of the respective basic groups, the abstracted H^+ ions could exchange with solvent water or become incorporated into the product, PPH_4, by way of internal return.

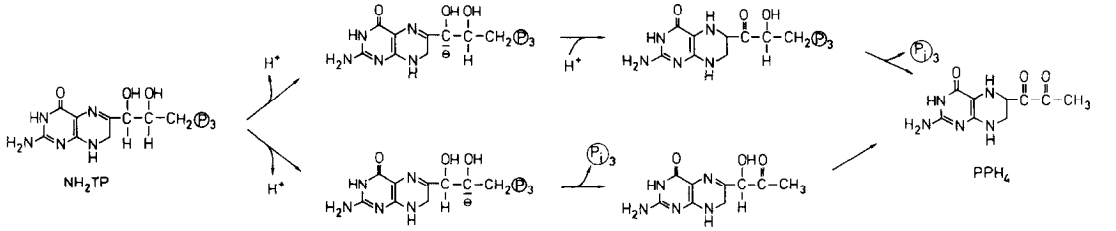

Fig. 2. Hypothetical reaction mechanisms for PPH_4 synthase (7)

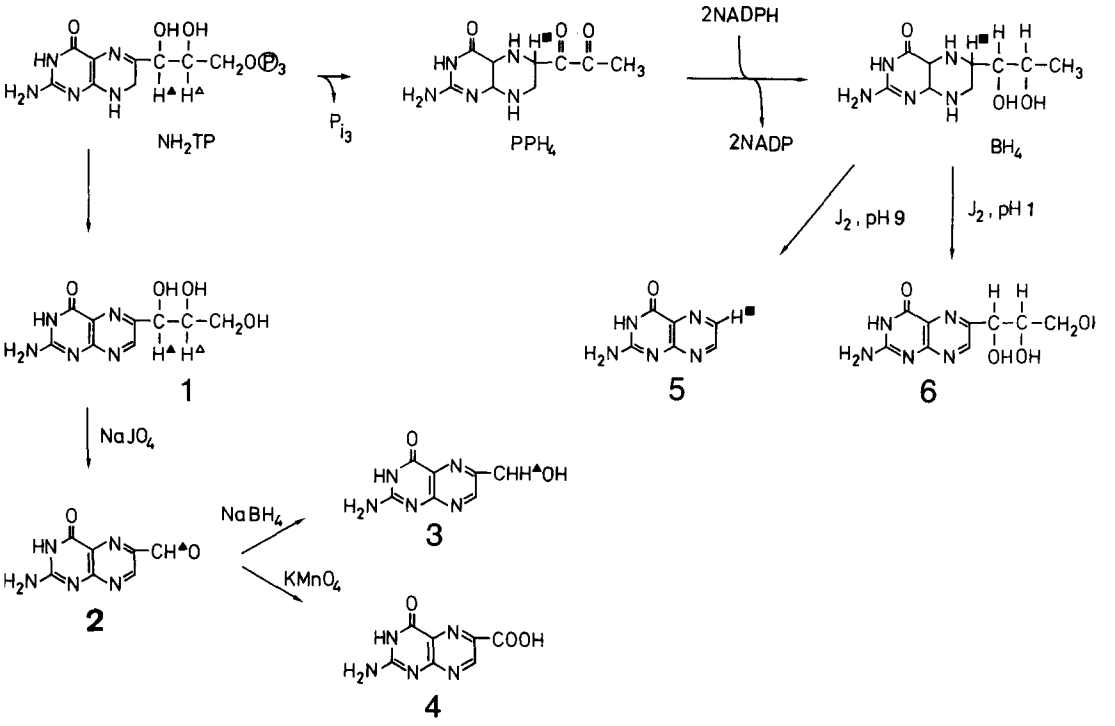

Fig. 3. Degradation of enzyme substrates and products

We have studied the fate of the H-atoms at C-1' and C-2' using [1'-^{3}H]- and [2'-^{3}H]-NH_2TP as substrates.

[1'-^{3}H]-NH_2TP and [2'-^{3}H]-NH_2TP were obtained by treatment of [3'-^{3}H]-GTP and [4'-^{3}H]-GTP with GTP cyclohydrolase I from *Escherichia coli*. In order to confirm the position of the ^{3}H labels, samples of labeled NH_2TP were treated with iodine and alkaline phosphatase (15) yielding neopterin (**1**) which was isolated by HPLC (Fig. 3). Treatment with periodate afforded pterin 6-aldehyde (**2**) which was converted to 6-hydroxymethylpterin (**3**) (by reduction with sodium borhydride) or to pterin 6-carboxylic acid (**4**) (by permanganate oxidation). The results of the degradation studies were as expected. Degradation of [1'-^{3}H]-NH_2TP yielded ^{3}H-labeled **3** and unlabeled **4**. Degradation of [2'-^{3}H]-NH_2TP yielded unlabeled **3** and **4**.

[1'-^{3}H]-NH_2TP and [2'-^{3}H]-NH_2TP were converted to PPH_4 by treatment with an enzyme preparation obtained from bovine adrenal medulla (11) (Fig. 3). This labile product was enzymatically reduced to BH_4 using NADPH as cosubstrate. Oxidation of BH_4 with iodine at pH 1 afforded biopterin (**6**) which was isolated by reversed phase HPLC (Fig. 3). Alternatively, treatment of enzymatically formed BH_4 with iodine at pH 9 (16) afforded pterin (**5**)

Table 1. Formation of tetrahydrobiopterin (BH_4) from ^3H-labeled NH_2TP

Substrate	Relative specific activity (%)[a]	
	Biopterin	Pterin
[1'-^{3}H]NH_2TP	0.6 ± 0.3	0.1 ± 0.1
[2'-^{3}H]NH_2TP	0.4 ± 0.1	0.1 ± 0.04

NH_2TP (obtained from GTP) was converted to BH_4 by a partially purified extract from bovine adrenal medulla. BH_4 was analyzed after conversion to biopterin and pterin, respectively.

[a] specific activity of biopterin or pterin/specific activity of NH_2TP x 100

which was again isolated by HPLC. The specific radioactivities of these products were measured. Results are shown in Table 1. The specific radioactivities of biopterin (**6**) and pterin (**5**) were less than 1 % as compared to the substrate, NH_2TP (determined as neopterin (**1**) after dephosphorylation and oxidation).

It has been shown that the H-atom in position 6 of BH_4 is retained in pterin obtained by iodine oxidation (17). Thus, any ^3H incorporated into the 6-position of BH_4 should appear in pterin. Any ^3H incorporated into the methyl group of PPH_4 should show up in biopterin. The data show the virtually complete elimination as opposed to internal return of both hydrogen atoms at position 1' and 2' of NH_2TP.

Subsequent experiments were designed to investigate whether PPH_4 synthase can catalyze the exchange of H-atoms at C-1' or C-2' of the substrate, NH_2TP, with solvent protons. To analyze this question, we incubated adrenal medulla extract with [1'-^{3}H]- or [2'-^{3}H]-NH_2TP and isolated the remaining NH_2TP at intervals. Iodine oxidation and enzymatic dephosphorylation yielded neopterin whose specific radioactivity was determined. The amount of tritium water formed was also determined.

The extent of the reaction was based on the consumption of the substrate, NH_2TP (determined after oxidation and dephosphorylation). The amount of BH_4 formed (determined after oxidation) was consistently lower than the amount of substrate consumed. This is not surprising in light of the known instability of PPH_4 and the involvement of two subsequent enzymatic reduction steps.

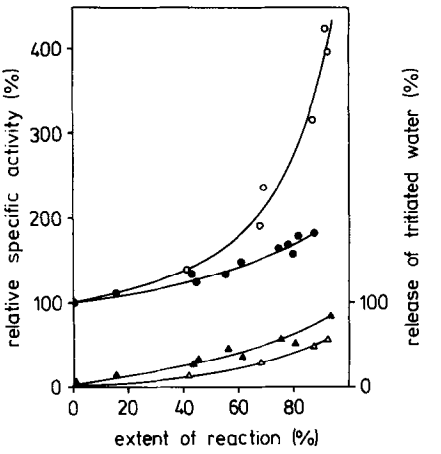

Fig. 4. Kinetic isotope effects in the formation of PPH_4 from NH_2TP. The specific radioactivity of remaining substrate, [1'-^{3}H]-NH_2TP (●) or [2'-^{3}H]-NH_2TP (○) and the release of tritium water from [1-^{3}H]-NH_2TP (△) and [2'-^{3}H]-NH_2TP (▲), were measured at intervals.

The specific radioactivity of the remaining [1'-^{3}H]-NH_2TP showed a substantial increase with progression of the enzyme reaction (Fig. 4). This increase represents a kinetic isotope effect of 2.6 ± 0.5. An increase of the specific activity of remaining substrate was also observed with [2'-^{3}H]-NH_2TP indicating a kinetic isotope effect of 1.5 ± 0.2. No evidence for an enzyme-catalyzed exchange of substrate hydrogen atoms with solvent protons was observed. The rate of formation of tritium water during the reaction is in full agreement with this interpretation.

DISCUSSION

The CH-bonds at C-1' and C-2' must be broken in the reaction from NH_2TP to PPH_4. We have shown that very little (less than 1 %) if any of the tritium released from the 1' or 2' position of NH_2TP is subsequently incorporated into PPH_4 and BH_4 by internal return. Thus it appears that the basic groups responsible for H^+ abstraction from NH_2TP are accessible to solvent.

Smith and coworkers (17) have shown earlier that the H atom at position 6 of BH_4 is introduced from solvent water. However, the mass spectroscopic technique used was probably less sensitive for the exclusion of partial internal return as compared to the present method.

In the formation of PPH_4, NH_2TP labeled with ^{3}H at position 1' or 2' reacts more slowly as compared to the unlabeled substrate (kinetic isotope effects of 2.6 ± 0.5 and 1.5 ± 0.2, respectively). No evidence for an enzyme-catalyzed exchange of the 1' or 2' H-atoms was found.

It should be noted that the isotope competition experiments described in this paper can not rule out a slow hydrogen exchange. They do show, however, that the rate of any potential exchange can not be rapid in comparison with the rate of product formation.

It is obvious that the mechanism of PPH_4 synthase must be complex, given the extensive remodeling of the substrate molecule. Due to the virtually complete absence of mechanistic background data it is not yet possible to interpret the observed kinetic isotope effects in detail. However, the data show that the breaking of the CH bonds represent energetically and kinetically relevant steps rather than a rapid preequilibrium. A more detailed investigation of this problem is under way.

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. We thank Drs. R. Switzer and B. Hove-Jensen for a subculture of *Escherichia coli* PH0561.

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