Characterization of a novel pterin intermediate formed in the catalytic cycle of tyrosine hydroxylase

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A novel pterin intermediate, in addition to the expected 4a-hydroxytetrahydrobiopterin (4a-OH-BH₄) and quinonoid dihydrobiopterin, was generated during catalytic turnover of tyrosine hydroxylase (TH) with tetrahydrobiopterin as the cofactor. Based on chromatographic, spectroscopic and stability properties its structure is proposed to be similar to the product formed by the non-enzymic conversion of synthetic 4a-OH-BH₄ [Bailey, Rebrin, Boerth and Ayling (1995) *J. Am. Chem. Soc.* **117**, 10203–10211]. This compound was tentatively described as a 4a-adduct of a side-chain hydroxy group, i.e. the O2',4a-cyclic-tetrahydrobiopterin (4a-Cyc-BH₄). The intermediate generated in the TH reaction has a UV spectrum which is similar to that of 4a-OH-BH₄, but elutes with a longer retention time (*t_R* = 1.69 min compared with 1.06 min) on reversed-phase chromatography. Its conversion into quinonoid dihydrobiopterin is catalysed by

pterin-4a-carbinolamine dehydratase (EC 4.2.1.96), although 4a-OH-BH₄ is the preferred substrate for that enzyme. A precursor–product relationship was demonstrated between 4a-OH-BH₄ and the putative 4a-Cyc-BH₄ intermediate. The apparent stability of this compound is dependent on pH as well as on the nature of the buffer ions. At pH 8.0 a large amount was generated in Hepes and Tris, but little in phosphate buffer. At pH 7.0 in Hepes (standard assay conditions) and Tris buffer the putative 4a-Cyc-BH₄, but no 4a-OH-BH₄, was observed. None of the intermediates was observed at pH 6.0. The accumulation of these intermediates in the absence of dehydratase has important implications for the assay of TH and phenylalanine hydroxylase activities, and is also compatible with a possible physiological role of the dehydratase in the synthesis of catecholamines *in vivo*.

INTRODUCTION

Aromatic amino acid hydroxylases such as tyrosine hydroxylase (TH; EC 1.14.16.2) and phenylalanine hydroxylase (PAH; EC 1.14.16.1) require the reduced pteridine cofactor (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) to activate dioxygen and hydroxylate the aromatic ring of the amino acid substrate (for reviews, see [1,2]). Based on steady-state kinetics of the TH-catalysed reaction a sequential mechanism has been proposed with an ordered binding of BH₄ as the first substrate, followed by dioxygen and L-tyrosine [3]. The cofactor product is released from the enzyme as 4a-hydroxytetrahydrobiopterin (4a-OH-BH₄) [4,5] and is subsequently dehydrated to quinonoid dihydropterin (q-BH₂) [6] before it is reduced by the NADH-dependent dihydropteridine reductase to regenerate BH₄ [7]. Dehydration occurs non-enzymically, but the reaction is also catalysed by an enzyme, pterin-4a-carbinolamine dehydratase (EC 4.2.1.96) [6,8] which has been shown to be important in the PAH-catalysed hydroxylation of L-phenylalanine in the liver [2,9]. In our previous studies on the catalytic mechanism of bovine TH and PAH, the reaction products of BH₄ were studied by reversed-phase HPLC and rapid-scanning spectroscopy [10]. In addition to the 4a-OH-BH₄, q-BH₂ and BH₂ species, a chromatographic peak with spectroscopic properties similar to 4a-OH-BH₄, but with a longer retention time (*t_R*), was observed. In the present study it is shown that this compound, formed in the hydroxylation reactions catalysed by both TH and PAH, is similar to the product reported by Bailey et al. [11], generated from a non-enzymic intramolecular cyclization of the synthetic 4a-OH-BH₄, i.e. the putative O2',4a-cyclic-tetrahydrobiopterin (4a-Cyc-BH₄). The conclusion is based on its chromatographic

and spectroscopic properties, as well as its function as a substrate for pterin-4a-carbinolamine dehydratase (dehydratase). Furthermore, a precursor–product relationship is demonstrated for the two pterin intermediates formed in the TH reaction, and the side-chain hydroxyl functions in the 6-position of BH₄ were found to be required for the formation of the putative 4a-Cyc-BH₄ intermediate. The apparent stability of the intermediate depends both on buffer ions and pH, and it is shown that under certain assay conditions the accumulation of this intermediate may lead to errors in the estimation of hydroxylase activity. Finally, the requirement for dehydratase in the TH-catalysed hydroxylation reaction is addressed.

MATERIALS AND METHODS

Materials

BH₄, (6*R*,*S*)-methyl-5,6,7,8-tetrahydropterin (6-MPH₄) and (6*R*,*S*)-5,6,7,8-tetrahydro-D-neopterin were purchased from Dr. B. Schirck's Laboratory (Jona, Switzerland).

Enzymes

Isoform 1 of recombinant human tyrosine hydroxylase (hTH1) was expressed in the pET system of *Escherichia coli* and purified to homogeneity as described previously [12]. Recombinant human phenylalanine hydroxylase (hPAH) was expressed in the pMAL system of *E. coli* and purified as described [13]. Recombinant human pterin-4a-carbinolamine dehydratase (dehydratase) was a generous gift from Dr. Sandro Ghisla, University of Konstanz, Germany.

Abbreviations used: BH₄, (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin; 4a-OH-BH₄, 4a-hydroxytetrahydrobiopterin; 4a-Cyc-BH₄, O2',4a-cyclic-tetrahydrobiopterin; q-BH₂, quinonoid dihydrobiopterin; 6-MPH₄, (6*R*,*S*)-methyl-5,6,7,8-tetrahydropterin; DTT, dithiothreitol; hPAH, recombinant human phenylalanine hydroxylase; hTH, recombinant human tyrosine hydroxylase; dehydratase, pterin-4a-carbinolamine dehydratase; *t_R*, retention time.

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Assay of TH activity and generation of pterin intermediates

The specific activity of hTH1 was assayed at pH 8.0 (Tris buffer) and pH 7.0 (Hepes buffer) as described [12]. The generation of pterin intermediates in the TH reaction was performed at 25 °C in a reaction mixture containing 100 mM Tris buffer (or the selected buffer, see the Results section), 0.5 mg/ml catalase, 1 mM L-phenylalanine or 100 μ M tyrosine, 10 μ M Fe(II), 1.44 μ M (subunit concentration) hTH1, 100 μ M BH₄ (or synthetic BH₄ analogues) and 1 mM dithiothreitol (DTT) to prevent accumulation of the quinonoid form, q-BH₂. TH was pre-incubated with the substrate (L-tyrosine or L-phenylalanine) for 5 min before the addition of tetrahydropterin. After a reaction period of 1–10 min, aliquots of the reaction mixture were injected directly into the HPLC system. The coupling efficiency of the reaction was measured essentially as described [14].

HPLC and spectroscopic characterization of tetrahydrobiopterin reaction products formed in the TH reaction

The pterin products generated in the TH reaction were separated by HPLC as described previously [10], using a 60 mm $\times$ 4.6 mm internal diameter (3 μ m particle size) C₁₈ reversed-phase column equipped with a 30 mm $\times$ 4.6 mm internal diameter C₁₈ pre-column (Hewlett Packard). The mobile phase contained 30 mM sodium acetate and 3% (v/v) methanol (pH 7.0). The flow rate was 1.0 ml/min. UV detection and absorption spectra were obtained by a 1040A photodiode array HPLC detector (6 mm pathlength).

RESULTS

Identification and characterization of a novel pterin intermediate formed in the TH reaction

At standard assay conditions, using L-tyrosine as the substrate, hTH1 had a specific activity of 113 ± 19 and 325 ± 6 nmol of L-DOPA/mg of protein per min in Tris/HCl buffer (pH 8.0) and Hepes buffer (pH 7.0) respectively, in the presence of 4a-OH-dehydratase. When an aliquot of the reaction mixture at pH 8.0 was applied to the HPLC column at $t = 2$ min, four chromatographic peaks related to the cofactor were observed (Figure 1); i.e. 4a-OH-BH₄ (peak 1, $t_R = 1.06$ min), q-BH₂ (peak 2, $t_R = 1.57$ min), the putative 4a-Cyc-BH₄ (peak 3, $t_R = 1.69$ min) and BH₄ (peak 4, $t_R = 1.88$). The four components were characterized on the basis of their spectroscopic properties as previously described [10]. It is seen from Figure 2 that the spectrum of peak 3 was almost identical to that of peak 1 (4a-OH-BH₄). The determination of the UV spectrum of the pterin component in peak 3 was, however, complicated by its co-elution with L-tyrosine as well as some contribution from the incompletely resolved peak of the quinonoid form, q-BH₂. To minimize these effects, L-phenylalanine was used as the substrate for TH [15] and a difference spectrum was obtained by subtracting the spectrum of peak 3 (Figure 1B) from the spectrum of peak 3 (Figure 1A). The former spectrum was obtained from a reaction mixture also containing dehydratase, which prevents the accumulation of the pterin intermediate (see below). The difference spectrum thus obtained was found to be almost identical to that of 4a-OH-BH₄ (Figure 2). The same main products were observed when PAH was used as the enzyme source instead of TH, and with L-tyrosine as the substrate for TH (results not shown). The concentration of 4a-OH-BH₄ generated after 2–4 min in the TH-catalysed reaction was found to be dependent on pH as well as the nature of the buffer ions in the reaction mixture (Figure 1), as previously described for synthetic and enzymically generated 4a-OH-tetrahydropterins [9,16]. Using Hepes or Tris buffers the

Figure 1 Tetrahydrobiopterin reaction products generated in the TH reaction as a function of pH

The reaction conditions are described in the Materials and methods section. After preincubation of hTH1 (1.44 μ M) for 5 min with 1 mM L-phenylalanine, the reaction was initiated by the addition of 100 μ M BH₄ and 1 mM DTT, and after 2 min the reaction mixture was analysed by HPLC. Peak identification: 1, 4a-OH-BH₄; 2, q-BH₂; 3, the putative 4a-Cyc-BH₄; 4, BH₄. (A) The reaction was performed in 100 mM Tris buffer, pH 8.0. (B) As in (A), but with 2.5 μ M of dehydratase added 1 min before BH₄. (C) The reaction was performed in 100 mM Tris buffer, pH 7.0. (D) As in (C), but in the presence of 2.5 μ M dehydratase. The absorbance scale at 246 nm (continuous line) is indicated by the bar; the sensitivity at 400 nm (dotted line) was four times higher.

putative cyclic intermediate appeared to be more stable at pH 8.0 than at pH 7.0 (Figures 1A and 1C). However, in sodium phosphate buffer, pH 8.0, only the 4a-OH-BH₄ species was observed. At pH 7.0 in Hepes and Tris buffer no 4a-OH-BH₄ could be seen, but the other intermediate was present in significant amounts. None of the pterin intermediates was observed in Hepes buffer at pH 6.0. It should be noted that the net formation of the putative 4a-Cyc-BH₄ intermediate observed in our study is a combination of two effects, i.e. the actual stability of the compound and its rate of formation.

Figure 2 UV spectrum of the novel pterin intermediate compared with that of 4a-OH-BH₄

The UV spectrum of 4a-OH-BH₄ (dotted line) was obtained from peak 1, Figure 1(A) and the spectrum of the putative 4a-Cyc-BH₄ (continuous line) was obtained from peak 3, Figure 1(A) and corrected for the presence of L-tyrosine and q-BH₂ as described in the text.

Figure 3 Time course for the generation of tetrahydrobiopterin reaction products in the TH reaction

The TH reaction was performed as described in the Materials and methods section. After incubation for 1, 3, 5 and 10 min, the reaction mixture was analysed by HPLC. Symbols: ●, the putative intermediate 4a-Cyc-BH₄; ○, 4a-OH-BH₄; ▼, BH₄. The numbers represent the average of two experiments.

The apparent coupling ratio (i.e. mol of tyrosine formed: mol of q-BH₂ formed) of the TH reaction is also affected by the accumulation of pterin intermediates. Based on the reaction stoichiometry, this ratio should be 1.0 [1,5,14]. However, in the absence of dehydratase, and after 1 min incubation this ratio is about 1.8 at pH 8.0 in Tris buffer and under similar conditions 1.2 in phosphate buffer.

Precursor-product relationship between the two pterin intermediates

The time course for the generation of the two pterin intermediates in the TH reaction at pH 8.0 (Tris buffer) is shown in Figure 3.

Figure 4 4a-OH-BH₄ and the putative 4a-Cyc-BH₄ as substrates for dehydratase

The TH reaction was performed in 100 mM Tris buffer, pH 8.0, as described in the Materials and methods section. Increasing concentrations of dehydratase was added to the reaction mixture after 2 min incubation. The dehydratase was allowed to work for 30 s before the mixture was analysed by HPLC. Symbols: ●, the putative intermediate 4a-Cyc-BH₄; ○, 4a-OH-BH₄; ▼, BH₄. The numbers represent the average of two experiments.

Whereas a maximum level of 4a-OH-BH₄ was observed after about 1 min, followed by a rapid disappearance, the pterin component in peak 3 (Figure 1A) slowly increased and reached a maximum after 3–5 min. The amount of BH₄ decreased slightly during the first 5 min of the reaction, then increased as the two intermediates were non-enzymically converted into q-BH₂ and regenerated to BH₄ on reduction by DTT.

Effect of dehydratase on the generation of pterin intermediates

Both pterin intermediates were found to be substrates for the dehydratase. When the TH reaction was performed at pH 7.0 and pH 8.0 in the presence of a high concentration (2.5 μM) of dehydratase, none of the intermediates was accumulated (Figures 1B and 1D). Furthermore, 4a-OH-BH₄ was found to be a better substrate for the dehydratase than the putative 4a-Cyc-BH₄ intermediate (Figure 4). Thus, when both intermediates were generated in the TH reaction, 4a-OH-BH₄ revealed the most rapid conversion into q-BH₂ upon the addition of increasing concentrations of the dehydratase.

The generation of pterin intermediates in the TH reaction using BH₄ analogues

Physicochemical properties of the novel pterin intermediate have indicated that it may be a 4a-adduct of the side-chain hydroxy group in the 6-position of BH₄ [11]. Our studies with synthetic cofactor analogues support this proposal. Thus, using 6-methyl-tetrahydropterin as the cofactor in the TH reaction, no compound corresponding to the cyclic intermediate was observed by HPLC. On the other hand, (6*R,S*)-5,6,7,8-tetrahydro-D-neopterin, which also has OH-substituents on the side chain in the 6-position, formed an intermediate analogous to the putative 4a-Cyc-BH₄,

as observed by chromatographic and spectroscopic properties of the intermediates (results not shown).

DISCUSSION

Identification of the novel pterin intermediate as 4a-Cyc-BH₄

It has recently been shown that when synthetic 4a-OH-BH₄ is dissolved in aqueous buffer a novel pterin intermediate is formed in addition to the expected q-BH₂ [11,17]. Its structure was suggested to be a cyclic adduct generated by cyclization of the 4a-OH substituent with the side-chain hydroxy group in the 6-position of BH₄, i.e. an *O'*,4a-cyclic-tetrahydrobiopterin (4a-Cyc-BH₄) [11]. This conclusion was based on its chromatographic and spectroscopic behaviour, identification of its conversion product (q-BH₂) and its electrochemical activity [11]. Here we show that the intermediate formed during catalytic turnover of TH is similar to the putative 4a-Cyc-BH₄ formed by a non-enzymic conversion of synthetic 4a-OH-BH₄. Probably due to its low stability, our attempts to characterize this intermediate further by HPLC-mass spectrometry (LC-MS) have so far not been successful. The reaction products of BH₄, as generated in the catalytic turnover of TH and PAH, are outlined in Scheme 1.

Precursor-product relationship of the pterin intermediates

The precursor-product relationship between 4a-OH-BH₄ and the novel pterin intermediate was demonstrated following the generation of both pterin intermediates in the TH reaction as a function of time. After 1 min at pH 8.0 and at high turnover conditions most of the cofactor was recovered as 4a-OH-BH₄. The amount of this species decreased with time and a corresponding increase in the amount of the novel pterin intermediate was observed after 3–5 min, whereas the change in the amount of BH₄ was less pronounced.

Effect of pH and buffer ions on the apparent stability of the pterin intermediates

The stability of 4a-OH-BH₄ is dependent on both pH and buffer ions [9,16] with a minimum rate of dehydration to the quinonoid form around pH 8.0 and a high rate at pH 7.0. In the present study we have established that the generation of the putative cyclic intermediate in the TH reaction after 2–4 min is dependent on the nature of the buffer as well as pH. Thus, in Tris and HEPES buffers both intermediates were present in the reaction mixture at pH 8.0, while only the cyclic intermediate was seen at pH 7.0. In sodium phosphate buffer, pH 8.0, only 4a-OH-BH₄ accumulated, although trace amounts of the putative cyclic intermediate (peak 3 in Figure 1A) could be masked by the co-elution of L-tyrosine.

Scheme 1 Schematic presentation of the hydroxylation reactions catalysed by TH and PAH, including the novel intermediate 4a-Cyc-BH₄

Abbreviation: DOPA, L-3,4-dihydroxyphenylalanine.

This finding is in good agreement with the observation that the dehydration of the synthetic 4a-OH-BH₄ to the quinonoid form is about 10 times more rapid than its cyclization to 4a-Cyc-BH₄ in 40 mM potassium phosphate [11]. This finding may also explain why the putative 4a-Cyc-BH₄ was not observed by Davis and Kaufman [18] in their studies on the oxidation products of BH₄ in the PAH-catalysed reaction, since all experiments were performed in potassium phosphate buffer.

Implications for the kinetic properties and assay of aromatic amino acid hydroxylases

The accumulation of the products 4a-OH-BH₄ and the putative 4a-Cyc-BH₄ species during TH and PAH turnover at pH 8.0 has obvious implications for kinetic studies of the hydroxylases at this pH. Thus, it has been reported that the specific activity of rat PAH is markedly decreased when assayed with BH₄ at increasing enzyme concentrations at pH 8.0 and related to a marked increase in the apparent *K_m* for the cofactor [19]. This effect was abolished when dehydratase was present in the assay mixture and was not observed with 6-MPH₄ as the cofactor, and it may thus be explained by product inhibition or substrate depletion due to accumulation of a pterin intermediate, which is specific for BH₄. A related phenomenon has been observed in our measurements on the apparent coupling ratio of TH- and PAH-catalysed hydroxylation reactions (i.e. mol of hydroxylated product/mol of q-BH₂ formed). This value is up to 1.8 after 1 min incubation at pH 8.0 when BH₄ is the cofactor and dehydratase is not present in the reaction mixture. Thus, it is likely that the putative cyclic pterin intermediate is partly responsible for the inhibition of the PAH-catalysed reaction observed by Huang and Kaufman [19] and the apparent increase in coupling ratio found in the TH reaction of the present study. At pH 7.0 the non-enzymic dehydration of 4a-OH-BH₄ occurs sufficiently rapidly to prevent its accumulation, in contrast to the putative 4a-Cyc-BH₄ intermediate which was observed in both HEPES and Tris buffer at this pH. An accumulation of this intermediate will sequester the cofactor in an inactive and possibly inhibitory form and thereby affect the kinetic parameters. Thus, when selecting the experimental conditions for the assay of both TH and PAH activity, using BH₄ as the cofactor, one should avoid high turnover conditions or include an excess of dehydratase in the reaction mixture.

The importance of dehydratase activity during TH turnover *in vivo*

Previous studies have indicated that the dehydratase may be more important for the PAH- than for the TH-catalysed reactions. In rats only liver and kidney express high dehydratase activity, and are also the only tissues that express PAH activity (liver $\geq$ kidney) [20]. A mild form of hyperphenylalaninaemia, characterized by the urinary excretion of large amounts of the 7-substituted isomer of BH₄, is likely to be caused by an impaired dehydratase activity [21,22], but there have been no reports of abnormalities in catecholamine biosynthesis in these patients. For rat liver it has been estimated that after a dietary phenylalanine load the high PAH activity will cause almost all of the available cofactor to reside in the 4a-OH-BH₄ form if dehydration is attributed only to a non-enzymic mechanism [9]. Thus, the dehydratase may not only be necessary for regenerating the correct isomer of the cofactor, but it also plays a kinetic role in PAH turnover. Similar arguments are likely to be valid also in cells which co-express dehydratase [20] and high levels of TH [23] and have a high basal biosynthetic capacity of catecholamines. However, no direct experimental evidence has been provided.

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REFERENCES

- Kaufman, S. (1995) *Adv. Enzymol.* **70**, 103–220
- Kaufman, S. (1993) *Adv. Enzymol.* **67**, 77–264
- Fitzpatrick, P. F. (1991) *Biochemistry* **30**, 6386–6391
- Bailey, S. W., Weintraub, S. T., Hamilton, S. M. and Ayling, J. E. (1982) *J. Biol. Chem.* **257**, 8253–8260
- Dix, T. A. and Benkovic, S. J. (1985) *Biochemistry* **24**, 5839–5846
- Kaufman, S. and Fisher, D. B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 285–369. Academic Press, New York
- Kaufman, S. (1964) *J. Biol. Chem.* **239**, 332–338
- Lazarus, R. A., Benkovic, S. J. and Kaufman, S. (1983) *J. Biol. Chem.* **258**, 10960–10962
- Bailey, S. W., Boerth, S. R., Dillard, S. B. and Ayling, J. E. (1993) *Adv. Exp. Med. Biol.* **338**, 47–54
- Haavik, J. and Flatmark, T. (1987) *Eur. J. Biochem.* **168**, 21–26
- Bailey, S. W., Rebrin, I., Boerth, S. R. and Ayling, J. E. (1995) *J. Am. Chem. Soc.* **117**, 10203–10211
- Haavik, J., Le Bourdellès, B., Martínez, A., Flatmark, T. and Mallet, J. (1991) *Eur. J. Biochem.* **199**, 371–378
- Martínez, A., Knappskog, P. M., Olafsdottir, S., Døskeland, A. P., Eiken, H. G., Svebak, R. A., Bozzini, M. L., Apold, J. and Flatmark, T. (1995) *Biochem. J.* **306**, 589–597
- Davis, M. D. and Kaufman, S. (1991) *FEBS Lett.* **285**, 17–20
- Fukami, M. H., Haavik, J. and Flatmark, T. (1990) *Biochem. J.* **268**, 525–528
- Haavik, J., Andersson, K. K. and Flatmark, T. (1989) *Pteridines* **1**, 11–16
- Rebrin, I., Bailey, S. W., Boerth, S. R., Ardell, M. D. and Ayling, J. E. (1995) *Biochemistry* **34**, 5801–5810
- Davis, M. D. and Kaufman, S. (1989) *J. Biol. Chem.* **264**, 8585–8596
- Huang, C. Y. and Kaufman, S. (1973) *J. Biol. Chem.* **248**, 4242–4251
- Davis, M. D., Kaufman, S. and Milstien, S. (1992) *FEBS Lett.* **302**, 73–76
- Adler, C., Ghisla, S., Rebrin, I., Haavik, J., Heizmann, C. W., Blau, N., Kuster, T. and Curtius, H. C. (1992) *Eur. J. Biochem.* **208**, 139–144
- Davis, M. D., Kaufman, S. and Milstien, S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 385–389
- Haycock, J. W. (1993) *J. Neurochem.* **60**, 493–502