Phenylalanine Hydroxylase: Absolute Configuration and Source of Oxygen of the 4a-Hydroxytetrahydropterin Species†

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ABSTRACT: The formation of tyrosine from phenylalanine catalyzed by rat liver phenylalanine hydroxylase is coupled to the generation of a 4a-hydroxy adduct from the requisite tetrahydropterin cofactor. As indicated by its circular dichroism (CD) spectrum, the optical activity of the adduct generated from racemic 6-methyltetrahydropterin requires stereoselectivity of the oxygenation. The absolute configuration of this new stereocenter is 4a(*S*)-hydroxy-6(*RS*)-methyltetrahydropterin by analogy to the CD spectrum of one of the four stereoisomers of 5-deaza-4a-hydroxy-6-methyltetrahydropterin. The source of the 4a-hydroxy oxygen is O_2, as demonstrated by the observation of a ^{18}O-induced ^{13}C shift in the ^{13}C NMR spectrum of the adduct when generated from [4a-^{13}C]-6-methyltetrahydropterin and $^{18}O_2$.

The conversion of phenylalanine to tyrosine catalyzed by phenylalanine hydroxylase (PAH)[1] requires a tetrahydropterin as an electron source. PAH oxygenates the tetrahydropterin during turnover to an unstable species that decays rapidly to an oxidized quinonoid dihydropterin (Kaufman, 1975), as shown in Scheme I for the synthetic cofactor 6-methyltetrahydropterin (6-MPH_4). This species has been identified as the carbinolamine 4a-hydroxy-6-methyltetrahydropterin (4a-OH-6-MPH_4), by UV and NMR spectroscopy (Lazarus et al., 1981, 1982a). Elucidation of the configuration at the 4a-position would further define the geometric requirements of the oxygenation. We have obtained direct evidence for the stereoselectivity of oxygen addition to racemic 6-MPH_4 by recording the CD spectrum of the 4a-OH-6-MPH_4 generated under stabilizing conditions and have elucidated the absolute configuration of the 4a stereocenter.

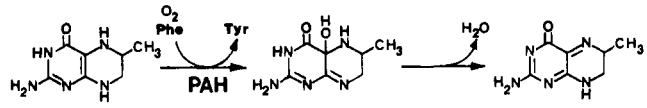
The chemical structure of the hydroxylating species generated by PAH is unknown. We have now demonstrated that the hydroxyl group of 4a-OH-6-MPH_4 is derived from O_2. A ^{18}O–^{13}C NMR isotopic shift is observed when 4a-OH-[4a-^{13}C]-6-MPH_4 is generated in an atmosphere 50% enriched in $^{18}O_2$. This result implicates the hydroxyl of 4a-OH-6-MPH_4 as being directly derived from the atom of O_2 not incorporated into tyrosine, thereby defining a narrow range of structural possibilities for the hydroxylating species of PAH.

Materials and Methods

Doubly distilled deionized water was used throughout. PAH was purified from rat livers through step IID of the method of Shiman et al. (1979), and had a specific activity of 6.2 μmol of tyrosine min^{-1} mg^{-1}. 6-MPH_4 was prepared from 6-methylpterin (Storm et al., 1971) by catalytic hydrogenation over 10% Pd/C. 5-Deaza-6-methyltetrahydropterin was prepared by Moad et al. (1979). [4a-^{13}C]-6-MPH_4 was prepared by Lazarus et al. (1982). 2(S),3(*S*)- and 2(R),3-(*R*)-di-*O*-benzoyltartaric acid were from K and K Laboratories (Plainview, NY), and DL-phenylalanine was from Mann Research Laboratories (New York, NY).

CD spectra were recorded on a Jasco J-20 spectropolarimeter, UV spectra on a Varian/Cary 118 or 219 spectrophotometer, NMR spectra on a Bruker 360-MHz spectrometer, and optical rotations on a Perkin-Elmer 241 polarimeter. HPLC separations were performed with a Waters 6000A pump, U6K injector, 440 UV detector (280 nm), a Whatman ODS-3 reverse-phase column (9.4 mm × 50 cm), and a Hewlett-Packard 3390A integrator. GC–MS was performed on a Finnigan 3200, 9500, 6000 system. ^{13}C NMR spectra were obtained on a Bruker Instrument Inc. WM-360 spectrometer operating at 90.56 MHz. The characterization spectra were proton decoupled and were measured with 16K data points, a sweep width of 9804 Hz (1.2 Hz/point), a 70° radio-frequency pulse, and an overall repetition rate of 0.84 s. Approximately 250 scans were acquired for each spectra. The spectra were resolution enhanced with an exponential function (LB = 0.5). The isotopic shift analysis spectra were proton decoupled and were measured with 16K data points, a sweep width of 1000 Hz (0.125 Hz/point), a 70° radio-frequency pulse, and an overall repetition rate of 8.0 s. Approximately 200 scans were acquired for each spectra. The spectra were resolution enhanced with a Gaussian window function (GB = −0.025, LB = −1.75). The chemical shifts were relative to an internal MeOH standard (δ = 49.0) with downfield being positive.

Scheme I



4a-OH-6-MPH_4 was generated at 4 °C for the CD experiment by adding 0.15 μmol of 6(*RS*)-MPH_4 to 1.5 mL of a solution containing 20 mM Tris, pH 8.5, 0.26 mg of PAH, 1.0 mM DL-phenylalanine, and 1.67 mM EDTA that had preincubated for 15 min. When the molar ellipticity at 290 nm had reached a minimum (after 2.5 min), the PAH was filtered out through a Millex-PF (0.8-μm) cellulose ester membrane (Millipore) and the CD spectrum recorded at a scan rate of 20 nm min^{-1}, time constant of 4 s, and slit width of 2 mm. Removal of PAH was confirmed by the disap-

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[1] Abbreviations: PAH, phenylalanine hydroxylase; 6-MPH_4, 6-methytetrahydropterin; 4a-OH-6-MPH_4, the 4a-hydroxy adduct of 6-MPH_4; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; GC–MS, gas chromatography–mass spectrometry; CAT, catalase.

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pearance of an intense negative CD signal at 230 nm. The concentration of the 4a-OH-6-MPH_4 was determined after correction for the remaining 6-MPH_4 and the rate of dehydration to quinonoid dihydropterin. Slight excesses of D-phenylalanine and L-tyrosine generated during the reaction did not contribute to the CD spectrum, since the base line was regained after complete 4a-OH-6-MPH_4 dehydration. Assays for 6-MPH_4 and tyrosine conducted during this reaction were as described by Lazarus et al. (1982a). The rate of 4a-OH-6-MPH_4 dehydration was measured by monitoring the change in molar ellipticity at 290 nm. The same rate was obtained when PAH was not filtered from solution.

Resolution of 5-deaza-6(*RS*)-methyltetrahydropterin was achieved by dissolving the free base in a minimal amount of hot ethanol and adding an equimolar amount of 2(*S*),3(*S*)-di-*O*-benzoyltartaric acid. The salt of 5-deaza-6(*R*)-methyltetrahydropterin crystallized out preferentially, and the partially resolved 6*S* isomer was recovered by evaporation of the mother liquor. This technique had been used by Armarego (1979) to resolve the 6-MPH_4 enantiomers. Tartrate was removed by dissolving the salts in a minimal amount of trifluoroacetic acid and precipitating out the trifluoroacetate salts of the deazapterins with methanol. After four recrystallizations, the enantiomer exhibited $[\alpha]_{435}$–25.0° in 2 N HCl (Armarego, 1979). The other enantiomer, recovered from the mother liquor after three successive recrystallizations, displayed $[\alpha]_{436}$ 15.0° (c = 50 g/100 mL of trifluoroacetic acid).

The 4a-hydroxy adducts of the resolved enantiomers were prepared by the method of Moad et al. (1979), and the ratio of trans to cis isomers in the resulting mixture was 3 to 2 as determined by ^1H NMR spectroscopy. Separation of these diastereomers by HPLC was modified from Moad et al. (1979). The mobile phase consisted of methanol/water/sodium dodecyl sulfate/sulfuric acid (45:55:0.02:0.04 v/v/w/v). Solutions of the diastereomers in 88% formic acid were injected at a flow rate of 4 mL min^{-1}. Peak fractions were collected, and the CD spectra were recorded directly in the mobile phase. Isomer concentrations in the collected fractions were determined from UV spectra. Aliquots of the collected fractions were reinjected onto the HPLC system to determine the success of the separations. HPLC assignments were confirmed by recording CD spectra of the crude diastereomeric mixtures whose predominantly trans composition had been verified by ^1H spectroscopy.

4a-OH-[4a-^{13}C]-6-MPH_4 was generated for the NMR experiment under conditions slightly modified from Lazarus et al. (1982a). Two 0.7-mL solutions of 0.04 M Tris, pH 8.5, were prepared at 4 °C. One contained 2.5 mg of PAH, 0.93 mg of Phe, and 0.7 mg of CAT and was saturated with $^{16}O_2$, while the other contained 0.85 mg of [4a-^{13}C]-6-MPH_4 and was saturated with $^{18}O_2$. After a 15-min preincubation, the enzyme solution was added to the freshly prepared 6-MPH_4 solution at 4 °C to initiate PAH turnover. Concentrations of reaction components were as follows: PAH, 36 μM; Phe, 4.0 mM; [4a-^{13}C]-6-MPH_4, 2.2 mM; O_2, 2.2 mM. After 2.5 min, an assay for remaining tetrahydropterin (Lazarus, 1982a) indicated that 75% had been oxidized, thus the unfiltered solution was added to 0.93 mL of CD_3OD at −30 °C followed by recording of the ^{13}C NMR spectrum at that temperature. The presence of 4a-OH-[4a-^{13}C]-6-MPH_4 in the incubation was confirmed by assaying a small aliquot for the decay of the adduct's characteristic spectrum (Lazarus et al., 1981).

The ^{18}O content of the tyrosine formed in the NMR experiment was quantitated by GC–MS. After filtration to remove enzyme, 0.2 mL of the incubation solution was evaporated in vacuo and treated with 1.0 mL of *n*-butyl alcohol/4 N HCl at 100 °C followed by evaporation and treatment with 1.0 mL of trifluoroacetic anhydride at 140 °C (Kaiser et al., 1974). An aliquot was injected onto a 6-ft, 3% OV-17 on Chromosorb W-HP column and eluted with standard gas flows using a temperature gradient of 100–250 °C at 5 °C/min. The *N*-trifluoroacetyl *n*-butyl ester of tyrosine eluted at 14.5 min and chromatographed with the same retention time as a standard of this compound, prepared and analyzed independently. The mass spectrum of the standard contained major ions at m/e 328, 316, 260, and 203, all diagnostic of derivatized tyrosine (Kaiser et al., 1969). The mass spectrum of the tyrosine from the NMR experiment contained the above peaks plus (m/e + 2) peaks of approximately equal intensity, demonstrating the partial incorporation of one atom $^{18}O_2$ into this compound. The ^{18}O:^{16}O ratio for the hydroxyl was quantitated in multiple runs from the ratio of ions at m/e 203 and 205 after appropriate corrections for carbon isotopes as these ions were the most abundant in the mass spectrum of derivatized tyrosine.

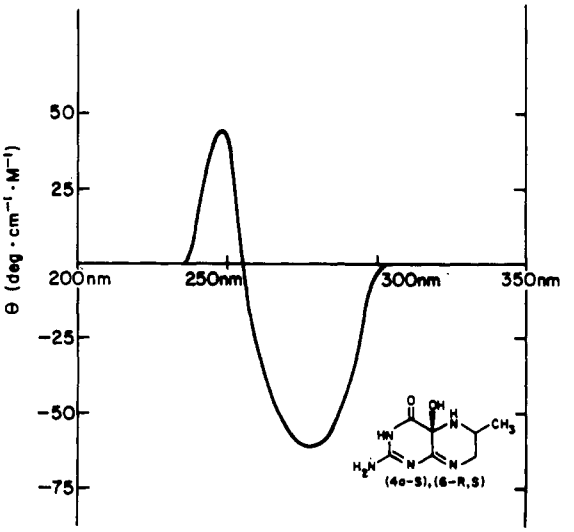

FIGURE 1: CD spectrum of 4a-OH-6-MPH_4 in 20 mM Tris, 4 °C.

Results and Discussion

The observation of a characteristic CD spectrum for the enzymically generated 4a-OH-6-MPH_4 (see Figure 1) clearly indicates that the hydroxyl group is attached predominantly to one face of the bicyclic ring system. The species whose CD spectrum is shown can be identified as 4a-OH-6-MPH_4 by its kinetic properties. Generation of this species in Tris buffer at 4 °C and pH 8.5 yields a reaction progress curve (when monitored by CD) having a first-order decay constant of 0.042 min^{-1}. This corresponds exactly to the rate constant predicted by the Arrhenius relation derived for the carbinolamine dehydration under these conditions (Lazarus et al., 1982a). Assays for the remaining 6-MPH_4 and accumulated tyrosine after generation of the CD spectrum shows that about 90% of the pterin is oxidized and that the anticipated tyrosine has formed. This result requires that both 6-MPH_4 enantiomers be utilized by PAH, and the oxygenation stereochemistry is therefore independent of the configuration at the 6-position (i.e., the hydroxy adds both cis and trans to the 6-methyl group). Also, if the enzyme had oxygenated one 6-enantiomer faster than the other[2] but always exclusively either cis or trans

[2] Studies with the analogous tetrahydrobiopterin isomers have indicated that both bind to PAH equally well but that one is utilized almost 4 times as fast as the other (Bailey & Ayling, 1978).

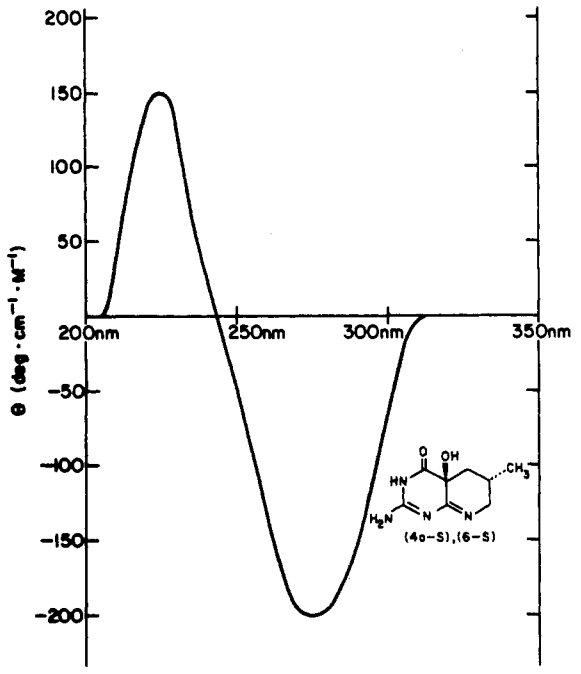

FIGURE 2: CD spectrum of 5-deaza-4a(*S*)-hydroxy-6(S)-methyltetrahydropterin in methanol/water/sodium dodecyl sulfate/sulfuric acid (45:55:0.02:0.04 v/v/w/v), 23 °C.

to the methyl group, then the rate of CD signal decay in the presence of PAH should coincide with the oxygenation rate for the slow enantiomer and not with the dehydration rate.

As shown in Figure 2, the CD spectrum of a 4a-hydroxydeazapterin with the 4a*S* configuration has the same shape as that of the enzymically generated 4a-OH-6-MPH_4. The diastereomer with a 4a(*S*)-hydroxy but with a 6(*R*)-methyl configuration has a similar CD spectrum (not shown), so the 6-methyl group has no influence on the signs of the Cotton effects. Therefore, the 4a*S* configuration predominates in the 4a-OH-6-MPH_4, giving rise to the spectrum of Figure 1. The CD magnitudes in Figures 1 and 2 cannot be compared directly, since the UV spectrum of the deazapterin in the HPLC mobile phase (λ_{max} 225, 280) is significantly different from that in Tris buffer (λ_{max} 245, 290). In Tris buffer at pH 8.5 where its UV spectrum is very similar to that of 4a-OH-6-MPH_4 (Lazarus et al., 1981), the hydroxy adduct of the deazapterin is unstable and has CD magnitudes roughly half of those reported here.

Absolute configurations for the deazapterins were assigned as follows. Optical rotations of the resolved 5-deaza-6-methyltetrahydropterin enantiomers were compared with those reported for the resolved 6-MPH_4 enantiomers. Since the chiral carbon in the deazapterin forms part of a conformationally labile ring, it was necessary to demonstrate that it is conformationally similar to 6-MPH_4 in order to confidently assign the absolute configuration. An analysis of ^1H NMR coupling constants indicated that both the deazapterin (Moad et al., 1979) and 6-MPH_4 (Weber & Viscontini, 1975) adopt pseudo chair conformations with the methyl groups equatorial. These resolved deaza enantiomers were then treated with performic acid to yield the corresponding 4a-hydroxy adducts, with the hydroxyl group adding preferentially trans to the 6-methyl group (Moad et al., 1979) as determined by ^1H NMR. The resulting diastereomeric pairs were separated by HPLC and identified by their elution peak size, and the 4a configuration was inferred from its known geometrical relationship to the established 6-position. These assignments were confirmed by also recording CD spectra of the predominantly

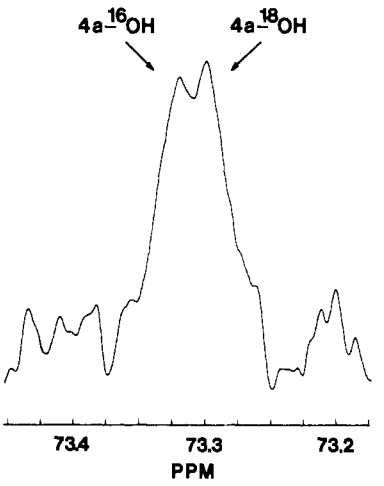

FIGURE 3: ^{13}C NMR spectrum of 4a-OH-[4a-^{13}C]-6-MPH_4 generated in an atmosphere 50% enriched in $^{18}O_2$.

trans diastereomeric mixtures.

Figure 3 shows the proton-decoupled ^{13}C NMR signal of 4a-OH-[4a-^{13}C]-6-MPH_4 when generated by PAH in an atmosphere approximately 50% enriched in $^{18}O_2$. A 0.023 ppm upfield shift is noted, diagnostic of an [^{18}O]hydroxyl group bonded to a tertiary carbon (Risley et al., 1980; Vederas, 1980). Relative peak heights are approximately 1:1 for ^{18}O:^{16}O. After the solution is warmed to 25 °C for 3 h, the 4a adduct signals at 73.3 ppm were replaced by a single signal at 101.5 ppm, indicative of 4a adduct dehydration and rearrangement to 6-methyl-7,8-dihydropterin (Lazarus et al., 1983). The $^{18}O_2$ content of the atmosphere was quantitated by determining the ^{18}O incorporation into the hydroxyl group of tyrosine (Kaufman et al., 1962) by GC–MS (Kaiser et al., 1974). The ^{18}O:^{16}O ratio of 52:48 seen in the hydroxyl group of tyrosine confirms that O_2 activation by PAH occurs without exchange of either oxygen with the water medium. Bailey et al. (1982) also have demonstrated the incorporation of $^{18}O_2$ into a diaminohydroxypyrimidinone product when a triaminopyrimidine is used as a cofactor for PAH.

The observation that 4a-OH-6-MPH_4 released from PAH is indeed optically active indicates that the binding of 6-MPH_4 depends on its planar orientation and that oxygen addition is directed to one side of this planar ring system. It is of interest to note that our configurational assignment at the 4a-position corresponds to a cis geometry with respect to the 6-position of "naturally occurring" tetrahydropterins, i.e., the isomers produced from the stereospecific reduction of 7,8-dihydrobiopterin and 7,8-dihydro-6-methylpterin by dihydrofolate reductase (Hasegawa et al., 1979). The fact that a discrete configuration is observed at C-4a rules against a rapid equilibrium between ring-closed and open pterin forms resulting from bond cleavage between C-4a and N-5. Such an equilibrium is conceivable given the results reported with triaminopyrimidones as cofactors for PAH (Bailey & Ayling, 1978) where the 4-amino function is substituted by a hydroxyl upon turnover.

Our demonstration of O_2 as the source of the hydroxyl of 4a-OH-6-MPH_4 defines a narrow range of possible structures for the hydroxylating species of PAH. Prior results (Lazarus et al., 1981, 1982a) strengthened by the present evidence disfavor a ring-opened carbonyl oxide as the hydroxylating species. Presuming that the nonheme iron is involved in the hydroxylation event, a pterin–O_2–iron(II) structure (Figure 4) emerges as a possible intermediate in PAH turnover (Lazarus et al., 1982c; Benkovic et al., 1984). Transfer of oxygen

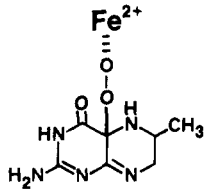

FIGURE 4: Structure of a possible intermediate in the activation of oxygen by PAH.

directly into phenylalanine could occur at this stage. Alternatively, formation of a peroxypterin species analogous to the bacterial flavin-requiring aromatic amino acid hydroxylases (Entsch et al., 1976) or a $[Fe{=}O]^{2+}$ formed by heterolytic cleavage of the peroxy bond might occur to generate the hydroxylating species. The latter is reminiscent of the hydroxylating species implicated in the catalytic cycle of cytochrome P-450 oxidase (White & Coon, 1980), although formally the iron is at a unit lower oxidation state. The putative structure in Figure 4, therefore, borrows elements from both of these enzyme types since simplistically the ease of substrate hydroxylation for PAH is intermediate. Experiments to define further the structural and energy requirements for the hydroxylating species of PAH are in progress.

ACKNOWLEDGMENTS

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Registry No. PAH, 9029-73-6; 6-MPH_4, 942-41-6; 4a-OH-6-MPH_4, 83387-39-7; 5-deaza-4a(*S*)-hydroxy-6(*S*)-methyltetrahydropterin, 96192-52-8.

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