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Mechanism of Oxygen Activation by Tyrosine Hydroxylase†

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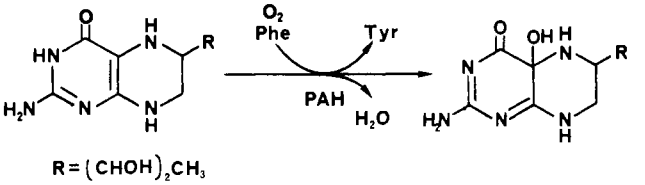
ABSTRACT: The mechanism by which the tetrahydropterin-requiring enzyme tyrosine hydroxylase (TH) activates dioxygen for substrate hydroxylation was explored. TH contains one ferrous iron per subunit and catalyzes the conversion of its tetrahydropterin cofactor to a 4a-carbinolamine concomitant with substrate hydroxylation. These results are in accord with shared mechanisms of oxygen activation by TH and the more commonly studied tetrahydropterin-dependent enzyme phenylalanine hydroxylase (PAH) and strongly suggest that a peroxytetrahydropterin is the hydroxylating species generated during TH turnover. In addition, TH can also utilize H_2O_2 as a cofactor for substrate hydroxylation, a result not previously established for PAH. A detailed mechanism for the reaction is proposed. While the overall pattern of tetrahydropterin-dependent oxygen activation by TH and PAH is similar, the H_2O_2-dependent hydroxylation performed by TH provides an indication that subtle differences in the Fe ligand field exist between the two enzymes. The mechanistic ramifications of these results are briefly discussed.

The tetrahydrobiopterin (BPH_4)[1]-dependent mixed-function oxidase tyrosine hydroxylase (TH) catalyzes the formation of DOPA from tyrosine and serves as the control point in catecholamine biosynthesis (Kaufman & Kaufman, 1984). While long presumed to be related to (if not identical with) the more extensively studied tetrahydropterin-dependent enzyme phenylalanine hydroxylase (PAH), firm evidence addressing this assumption has only recently become available due to the extreme difficulty in purifying sufficient quantities of TH for biochemical analysis. It is established that, while the enzymes share a significant (48%) sequence homology (Ledley et al., 1985; Dahl & Mercer, 1986), cofactor requirements, and characteristics of cofactor utilization, important differences exist as to subunit molecular weights and the mechanisms by which they are regulated in vivo (Shiman, 1985; Kaufman & Kaufman, 1985). The key question, whether TH activates oxygen by the same mechanism as does PAH, has been mainly unexplored.

Scheme I



In this paper, we examine the nature of oxygen activation by TH using our previous results with PAH as a guide. During

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[1] Abbreviations: BPH_4, L-*erythro*-tetrahydrobiopterin; TH, tyrosine hydroxylase; DOPA, L-3,4-dihydroxyphenylalanine; PAH, phenylalanine hydroxylase; 6-MPH_4, 6-methyltetrahydropterin; Tris, tris(hydroxymethyl)aminomethane; BPH_2, 7,8-dihydrobiopterin; EDTA, ethylenediaminetetraacetic acid; *m*-CPBA, *m*-chloroperoxybenzoic acid.

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catalytic turnover, PAH oxygenates its tetrahydropterin cofactor to a 4a-carbinolamine (Scheme I) concurrent with the hydroxylation of phenylalanine (Lazarus et al., 1981, 1982), a reaction that requires the presence of reduced iron in the enzyme. The 4a-carbinolamine dehydrates spontaneously to the final observed product of PAH turnover, quinonoid dihydropterin (BPH_2). The results to date are most consistent with a peroxytetrahydropterin as the hydroxylating intermediate of PAH (Dix et al., 1985; Dix & Benkovic, 1985; Benkovic et al., 1986). We now mechanistically link TH to PAH by demonstrating that TH contains one Fe^{2+} per subunit and converts BPH_4 to its 4a-carbinolamine concurrent with the hydroxylation of tyrosine. Most significantly, we demonstrate that H_2O_2 may be substituted for BPH_4 as a cofactor for tyrosine hydroxylation by TH. We discuss this alternative mechanism of oxygen activation that, while unique to this enzyme, provides further chemical plausibility for peroxytetrahydropterin as the hydroxylating species generated during TH and, by inference, PAH turnover.

Experimental Procedures

Materials

TH was purified to homogeneity from cultured rat pheochromocytoma (PC12) cells by the method of Kuhn.[2] 6-(*RS*)-BPH_4 and 6(*RS*)-MPH_4 were prepared by dissolving 50 mg of the corresponding dihydropterins in 50 mL of 6 N HCl containing 25 mg of palladium on carbon and hydrogenation under 40 atm of H_2 with shaking for 8 h. Tetrahydropterins were recovered as solids after filtration to remove catalyst followed by lyophilization. L-[3,5-^{3}H]Tyrosine (55.7 Ci/mmol) was purchased from New England Nuclear. Scintillation cocktail was Scintiverse II (Fisher). All other biochemicals were purchased from standard supply houses at the highest available purity. Distilled, deionized water was used in the preparation of all buffers.

Methods

Assays. Kinetic assays were performed on a Gilford 252 spectrophotometer. UV and fluorescence spectra were obtained on a Perkin-Elmer 3840 diode array spectrophotometer and a Perkin-Elmer MPF-66 spectrofluorometer, respectively. Atomic absorption was performed on a Varian/Cary instrument equipped with a flame-ionization tower. Conditions were optimized and standard curves generated for each metal assayed. A Beckman LS 6800 scintillation counter and premixed cocktails were used to determine ^{3}H release from tyrosine.

TH concentration was determined by the Lowry method (Lowry et al., 1951). TH activity was monitored by using two assays, one for oxidation of the tetrahydropterin cofactor (Shiman et al., 1979) and one for hydroxylation of substrate (Guroff et al., 1967). Tetrahydropterin oxidation to quinonoid dihydropterin was monitored for BPH_4 at 244 or 334 nm ($\Delta\epsilon$ = 4370 and 2920 M^{-1} cm^{-1}, respectively) and for 6-MPH_4 at 334 nm ($\Delta\epsilon$ = 3200 M^{-1} cm^{-1}). ^{3}H release from [3,5-^{3}H]-tyrosine into H_2O monitored the rate of tyrosine hydroxylation. ^3H_2O was separated from [^{3}H]tyrosine by binding the latter to Dowex 50w × 8 (200–400 mesh) in a 6 × 0.5 cm column and eluting the former with excess H_2O. The ^3H_2O was then counted in a scintillation counter to determine the amount of product formation. TH was assayed for phenylalanine hydroxylase activity in three ways: (a) by the cofactor oxidation assay described above; (b) by monitoring hydroxylation of phenylalanine at 275 nm ($\Delta\epsilon$ = 1700 M^{-1} cm^{-1}, in the presence of dithiothreitol) (Shiman et al., 1979); and (c) by the nitrosonaphthol fluorescence assay for phenols (Waalks & Udenfriend, 1957). Conditions utilized for these experiments were 10 μM TH and 20, 100, or 1000 μM cofactor in 0.02 M Tris, pH 8.2, 25 °C, or in 0.02 M KP_i, pH 7.2, 25 °C.

Fe Redox State. The redox state of Fe–TH was determined by using ferrous- and ferric-selective chelators, *o*-phenanthroline and catechol. *o*-Phenanthroline binds Fe^{2+} with a $\Delta\epsilon_{510}$ of 11 000 M^{-1} cm^{-1} (Schilt, 1969) while catechol binds Fe^{3+} with a $\Delta\epsilon_{650}$ of ca. 5000 M^{-1} cm^{-1} (Rowe, 1984). No absorbance at the given wavelengths was noted for the binding of *o*-phenanthroline to Fe^{3+} and catechol to Fe^{2+}. The $\Delta\epsilon$ values were checked in the given buffers by using standard solutions of the metals as a control for solvent effects.

4a-Carbinolamine. Evidence for the conversion of BPH_4 to its 4a-carbinolamine during TH catalytic turnover was obtained by using kinetic methods, as previously described (Dix & Benkovic, 1985). Standard incubation conditions were 5.0 μM TH, 20.0 μM tyrosine, and 20 μM BPH_4 in 1.0 mL of 0.02 M Tris, pH 8.2, 25 °C, with enzyme turnover initiated by the addition of cofactor. When substrate hydroxylation was measured, [^{3}H]tyrosine (1.0 μCi/μmol) was substituted for tyrosine, and time points were taken by quenching aliquots into 1.0 M acetic acid to terminate enzyme activity. No increase in nonenzymatic ^{3}H release from [^{3}H]tyrosine occured under these quench conditions. TH was concentrated for these experiments by utilizing Centricon filters (Amicon) with no loss in enzymatic activity after 10–50-fold concentration when performed at 4 °C.

PAH does not increase the rate of 4a-carbinolamine dehydration in the absence of its stimulator protein (Lazarus et al., 1983). TH was also tested for this activity. 4a-Carbinolamine was generated by addition of 20 μM BPH_4 to a solution of 1.0 μM PAH, 1.0 mM Phe, and 24 μg/mL catalase in 0.02 M Tris, pH 8.2 at 25 °C. After the absorbance of the solution at 244 nm had maximized, diagnostic of maximal 4a-carbinolamine formation, TH was added to a concentration of 2.0 μM, and the A_{244} was monitored with time until all 4a-carbinolamine had disappeared.

Peroxide-Dependent Hydroxylation. Incubation conditions for hydroxylation of tyrosine by TH and H_2O_2 were 1.0 μM TH, 20 μM [^{3}H]tyrosine (1.0 μCi/μmol), and H_2O_2 in 1.0 mL of 0.02 Tris, pH 8.2, 25 °C. Hydroxylation was determined by quenching of aliquots into acetic acid and determining ^3H_2O release, as described above. Experiments in which BPH_4 was reacted with TH in the initial absence of tyrosine were performed by adding 4.0 μM BPH_4 to 4.0 μM TH in 1.0 mL of 0.02 M Tris, pH 8.2 at 25 °C. Complete oxidation of BPH_4 to BPH_2 prior to tyrosine addition was demonstrated by following (a) BPH_4 oxidation to BPH_2 at 344 nm (vide infra) or (b) the disappearance of BPH_4 by the dichlorophenolindophenol assay (Lazarus et al., 1982). After BPH_4 had oxidized, [^{3}H]tyrosine (1.0 μCi/μmol) was added to 10 μM, time points were taken, and the extent of hydroxylation was determined as described above.

Computer Simulations. Computer simulations to provide a check on experimentally determined rate constants were performed on an IBM 3033 processor complex running under OS/VS2 MVS. The simulations program employed was Chemical Reactions Analysis and Modelling Systems (CRAMS), an integrated system of Fortran and OS Assembler models designed to simulate and predict data (DeMaine, 1980).

Results

Metal Content of TH. The presence of Fe in TH purified from rat adrenal tissue has been inferred in the literature

[2] D. M. Kuhn, manuscript in preparation.

Table I: Metal Content of Tyrosine Hydroxylase

sample[a]	TH (μmol)[b]	Fe (μmol)[c]	Fe/enzyme[d]
A	0.221	0.213	0.96
B	0.061	0.068	1.11
C	0.102	0.100	0.98
D	0.093	0.087	0.94

[a] Samples A and B are peak fractions from the final step in one TH purification while samples C and D are peak fractions from a second TH purification (D. M. Kuhn, unpublished results). In all four samples, the enzyme is <95% pure, as judged by polyacrylamide gel electrophoresis. [b] Micromoles of TH per metal determination. All values are averages of three protein determinations (Lowry et al., 1951) with standard errors less than 5%. [c] Micromoles of Fe per determination. The values are averages of three determinations with standard errors less than 10%. [d] Ratio of column *c* to column *b*.

Table II: Interaction of TH with Ferrous- and Ferric-Selective Chelators[a]

experiment	Fe^{2+}·*o*-phe[b] (pmol)	Fe^{3+}·cat[b] (pmol)
TH (1.07 pmol)	1.02	0
TH (0.52 pmol)	0.45	0
TH (0.93 pmol) + H_2O_2 (10 pmol)	0	0.78
no TH[c]	0	0
TH (0.57 pmol), no chelators[d]	0	0

[a] All incubations contained *o*-phenanthroline (*o*-phe) (1 μmol) or catechol (cat) (1 μmol) in 0.02 M KP_i, pH 7.2, 25 °C. Absorbance determined both before and after addition of TH, with correction for dilution. All experiments performed in quadruplicate with standard errors less than 10% for all values reported. [b] Determined as described under Experimental Procedures and under Results. [c] No TH added to incubation. [d] No *o*-phenanthroline or catechol included in the incubation.

(Nagatsu et al., 1964; Petrack et al., 1968) by the demonstration of increased enzymatic activity after Fe addition to incubations, although an alternative role of Fe as a peroxide scavenger was suggested by Kaufman and Kaufman (1985). Evidence for the presence of 0.5–0.75 Fe/TH subunit was also provided by Hoeldtke and Kaufman (1977) for TH purified from bovine adrenal medulla. We have now obtained direct evidence for the presence of Fe in our TH preparation by atomic absorption spectroscopy. Table I demonstrates that TH contains 1.0 ± 0.05 atom of Fe per subunit, based on a subunit molecular weight for this enzyme of 62 000.[2] The presence of other redox-active metals was also evaluated. No significant amounts of Co, Cu, Mo, or Mn were detected. These results parallel our earlier studies in which PAH was demonstrated to contain one Fe per subunit, evaluated by iron removal and replacement and wet ashing studies (Gottschall et al., 1982).

Redox State of TH Iron. Initial evidence for the redox state of TH iron as ferrous was obtained by demonstrating that the ferrous-selective chelator *o*-phenanthroline binds in a 1 to 1 stoichiometry with enzyme. This is presented in Table II and was determined by monitoring the increase in absorbance at A_{510} of the *o*-phenanthroline–Fe^{2+} complex which only appears in the presence of TH and which can be correlated to the amount of TH added, assuming that each enzyme subunit contains one Fe (vide supra). No absorbance change at 510 nm was seen by adding enzyme buffer minus TH or by adding TH in the absence of *o*-phenanthroline. The chelating effect of *o*-phenanthroline parallels the complete loss of activity of the TH enzyme from bovine adrenals caused by α,α-dipyridyl (Taylor et al., 1969).

PAH is catalytically active in the ferrous state but is isolated in the ferric state (Marota & Shiman, 1984; Wallick et al., 1984). The enzyme is reduced to Fe^{2+} at the expense of reducing equivalents from tetrahydropterin (Marota & Shiman, 1984; Wallick et al., 1984) and can reoxidize to Fe^{3+} either in a spontaneous reaction or in the presence of peroxides (Dix & Benkovic, 1985; Benkovic et al., 1986). Further evidence for the redox state of TH as ferrous was obtained by demonstrating a hysteretic cycle between ferrous and ferric using the same oxidizing and reducing equivalents previously utilized with PAH. Treatment of TH with excess H_2O_2 gave a protein that showed no spectral changes at 510 nm in the presence of *o*-phenanthroline but could interact with the ferric chelator catechol (Table II). The interaction was inferred by the observation of a catechol–Fe^{3+} band with an extinction maximum at 650 nm as well as by inhibition of enzyme turnover noted in the presence of catechol. This inhibition did not occur if the enzyme was oxidized by H_2O_2 without catechol or if turnover was initiated in the presence of catechol but without prior enzyme oxidation. Finally, oxidation of TH with 1 equiv of H_2O_2 and treatment of the enzyme with excess tetrahydrobiopterin resulted in retention of greater than 90% activity even in the presence of catechol. This implys that, like PAH, TH can be reduced from Fe^{3+} to Fe^{2+} by its tetrahydropterin cofactor.

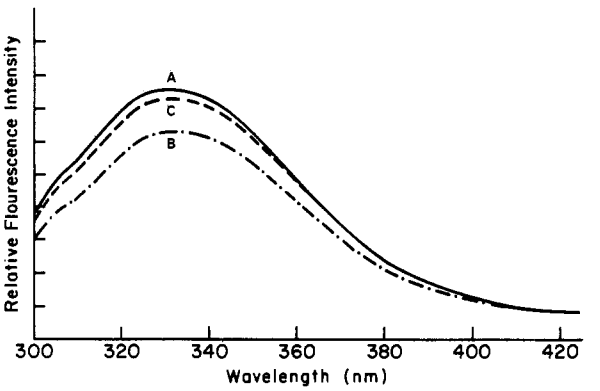


FIGURE 1: Fluorescence in TH vs. redox state of Fe. Scan A is the fluorescence emission spectrum of purified TH (1.0 μM) in 0.02 M Tris, pH 8.4, 25 °C, when excited at 275 nm. Scan B is the same as scan A except add 1.0 μM H_2O_2. Scan C is the same as scan B except add 1.0 μM BPH_4. Total time elapsed between each scan was 2 min.

Fluorescent changes of TH upon iron oxidation and reduction also match those previously established for PAH and confirm the results described above. Marota and Shiman (1983) showed that, when excited at 275 nm, PAH showed fluorescence with an A_{max} of 340 nm with an intensity sensitive to the redox state of its iron. About a 100% fluorescence enhancement is noted when PAH is reduced from Fe^{3+} to Fe^{2+}. Qualitatively similar results are seen when TH is subjected to a redox hysteretic cycle. Figure 1 demonstrates that native TH has a fluorescence spectrum similar to that reported for PAH. Treatment with 1 equiv of H_2O_2 results in a fluorescence quenching which can be abolished by the addition of 1 equiv of BPH_4. Although the magnitude of the quenching is significantly less, these results qualitatively match those for PAH. This cycle can be repeated 3–4 times with greater than 90% retention of TH activity.

4a-Carbinolamine of BPH_4 as an Intermediate in TH Catalytic Turnover. The instability of 4a-carbinolamines toward acid-catalyzed dehydration (for example, the 4a-carbinolamine of 6-MPH_4 has a k_{deh} of 0.01 s^{-1} in Tris buffer at pH 8.45, 25 °C; Lazarus et al., 1982) makes its identification and characterization as a reaction intermediate difficult. Conditions must be found in which its observed rate of formation is greater than its dehydration rate. While relatively easily done with PAH (Lazarus et al., 1982; Dix & Benkovic,

1985), the inherent low activity of TH and the propensity of BPH_4 to autoxidize, especially at basic pHs, required a precise juggling of conditions and intense concentrating of the enzyme to fulfill this requirement. To maximize assay conditions, we determined the rates of TH turnover and dehydration rates of the 4a-carbinolamine at different pHs and temperatures. The best case situation for detecting the 4a-carbinolamine as an intermediate in TH turnover is at pH 8.2 at 25 °C, at which 1.0 μM TH oxidizes BPH_4 (20 μM) with an initial velocity of 0.005 s^{-1} and the 4a-carbinolamine dehydrates with a first-order rate constant of 0.008 s^{-1}. These conditions were used in the experiments described below.

Our method for detection of the 4a-carbinolamine was to utilize UV absorbance changes in BPH_4 as it is oxygenated by TH to 4a-carbinolamine followed by dehydration to BPH_2. Two wavelengths, 244 and 334 nn, were monitored simulataneously during TH oxidation of a limiting amount of BPH_4. The first wavelength monitors absorbance changes due to oxygenation of BPH_4 to 4a-carbinolamine as well as overall oxidation of BPH_4 to BPH_2. The latter component can be factored out by using the absorbance changes at 334 nm, which monitor only oxidation of BPH_4 to BPH_2, as the 4a-carbinolamine does not absorb at this wavelength (Kaufman, 1975). This manipulation enables construction of reaction progress curves for formation and decay of 4a-carbinolamine during TH turnover. No other absorbance changes, such as for cofactor rearrangements or substrate hydroxylation, occur at these wavelengths (data not shown).

From the data presented above, we determined that a 2.0 μM concentration of TH, when run at pH 8.2 and 25 °C, should result in an observed rate of 4a-carbinolamine formation slightly greater than its dehydration rate. This presumes complete coupling between substrate hydroxylation and cofactor oxidation if, as with PAH, the 4a-carbinolamine forms only during turnover events resulting in substrate hydroxylation (Dix & Benkovic, 1985). Initial experiments, starting with 2.0 μM TH, 20 μM tyrosine, and 20 μM BPH_4, failed to exhibit any formation of 4a-carbinolamine. Simultaneous determination of the amount of tyrosine hydroxylated in these experiments revealed that only about 40–50% of the BPH_4 was being utilized in hydroxylation events. Since the 4a-carbinolamine forms only when tyrosine is hydroxylated (vide infra), its observed rate constant of formation was less than its rate of dehydration in these experiments, and no 4a-carbinolamine could be detected. The rest of the BPH_4 in these incubation is being oxidized directly to BPH_2 in a TH-dependent reaction which may or may not be autocatalytic (see Discussion). This appears to be an anomaly of running that reaction at basic pHs because controls demonstrated that greater than 95% of BPH_4 is used for hydroxylation when identical incubations were performed at pH 7.2, 25 °C.

Increasing the TH concentration to 5.0 μM enabled detection of the 4a-carbinolamine and the construction of reaction progress curves for its formation and decay (Figure 2). A first-order decay is noted (k_2 = 0.008 s^{-1}), identical with that obtained when PAH is utilized to generate the 4a-carbinolamine of BPH_4 under identical incubation conditions. This implies that TH possesses no carbinolamine dehydratase activity, an observation confirmed by adding TH to a solution of 4a-carbinolamine generated by PAH turnover (data not shown). Extrapolation of the first-order decay phase of 4a-carbinolamine to zero enables determination of total 4a-carbinolamine formed in the incubations. Starting with 20 μM BPH_4, the total amount of 4a-carbinolamine was 15.5 ± 1.0 μM. This compares to the total amount of tyrosine hydroxylation of 15.0 ± 0.5 μM in these experiments. Therefore, analogous to PAH (Dix & Benkovic, 1985), the 4a-carbinolamine forms concurrent with tyrosine hydroxylation. Table III demonstrates that, when incubations are assayed for the rate of both 4a-carbinolamine formation and tyrosine hydroxylation, the two quantities increase in parallel. Thus, DOPA and 4a-carbinolamine are the products of TH turnover of BPH_4, tyrosine, and O_2.

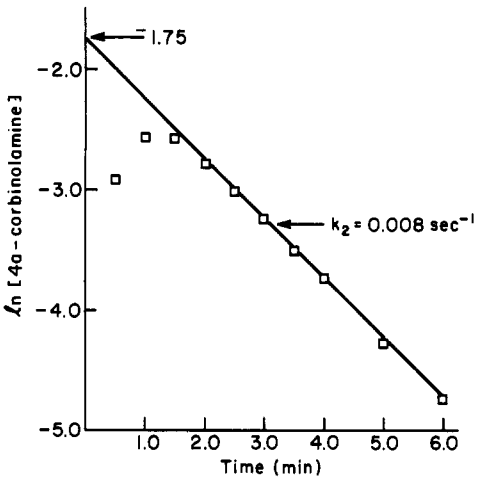


FIGURE 2: Reaction progress curve for the formation and decay of 4a-carbinolamine during TH turnover. Incubations contained 5.0 μM TH and 20 μM tyrosine in 1.0 mL of 0.02 M Tris, pH 8.2, 25 °C, and were initiated by addition of BPH_4 to 20 μM. Reactions were monitored at two wavelengths, 244 and 334 nm, which enabled the flux of 4a-carbinolamine to be determined at any given time point (see Results for a discussion). Reaction progress curves could be simulated by the CRAMS computer modeling system (DeMaine, 1980) and experimentally derived rate constants applied to the following minimal kinetic mechanism (cf. the introduction and Scheme I): $BPH_4 \xrightarrow{k_1}$ 4a-carbinolamine $\xrightarrow{k_2} BPH_2$.

Table III: Correlation of 4a-Carbinolamine and DOPA Formation by TH[a]

time[b] (s)	[DOPA][c] (μM)	[4a-OH][d] (μM)
0	0	0
30	6.2	6.2
60	9.3	9.9
90	13.3	11.6
120	14.3	13.2

[a] All incubations contained 5.0 μM TH and 20 μM tyrosine in 0.02 M Tris, pH 8.4, 25 °C, and were initiated by the addition of BPH_4 to 20 μM. [b] Time after BPH_4 addition. [c] Total DOPA formation at a given time. Average of duplicate experiments, determined by monitoring the release of ^3H into H_2O with [3,5-^{3}H]tyrosine (1.0 μCi/μmol) as the substrate (Experimental Procedures). [d] Total 4a-carbinolamine formation at a given time. This was calculated by using the CRAMS computer modeling system (DeMaine, 1980) and experimentally derived rate constants applied to the following minimal kinetic mechanism (cf. the introduction and Scheme I): $BPH_4 \xrightarrow{k_1}$ 4a-carbinolamine $\xrightarrow{k_2} BPH_2$. The residual rate of BPH_4 oxidation directly to BPH_2 induced by TH was subtracted out. Rate constants determined for these experiments were k_1 = 0.012 s^{-1} and k_2 = 0.008 s^{-1}.

Evaluation of Phenylalanine as a Substrate for TH. Various TH preparations are purported to have the ability to hydroxylate phenylalanine at a rate similar to that for the natural substrate (Ikeda, et al., 1965; Shiman, et al., 1971). Consequently, we evaluated our TH preparation for this activity utilizing spectrophotometric assays for substrate hydroxylation and cofactor oxidation as well as a fluorescence assay for tyrosine formation. Conditions were chosen so that one turnover event per enzyme molecule could be detected. However, we are unable to detect *any* phenylalanine hydroxylation by TH under these conditions using either cofactor

Table IV: Tyrosine Hydroxylation by TH and H_2O_2[a]

[TH] (μM)	[H_2O_2] (μM)	[hydroxylation][b] (μM)
0.5	1.0	0.25
0.5	10.0	0.61
0.5	100.0	0.09

[a] All incubations contained TH, 20 μM [3,5-^{3}H]tyrosine (1.0 μCi/μmol), and H_2O_2 in 0.02 M Tris, pH 8.2, 25 °C. [b] Determined by release of ^{3}H into H_2O as described under Experimental Procedures. Each experiment performed in quadruplicate with standard errors less than 5%.

BPH_4 or cofactor 6-MPH_4 at a variety of concentrations. In addition, no tetrahydropterin oxidation in excess of base line was detected. Controls demonstrated that phenylalanine is binding to the enzyme as it serves as a competitive inhibitor of tyrosine hydroxylation by TH.[2] Thus, phenylalanine does not appear to "uncouple" tetrahydropterin oxidation from substrate hydroxylation as does PAH in the presence of unnatural substrates such as *p*-chlorophenylalanine (Dix & Benkovic, 1985).

Oxidation of BPH_4 by TH in the Absence of Tyrosine. Experiments performed above revealed that a significant amount of BPH_4 oxidation in the absence of substrate hydroxylation occurs with TH at pH 8.2. To evaluate whether this cofactor oxidation was TH and/or tyrosine dependent, we measured the initial velocity of oxidation of 20 μM BPH_4 by 2.0 μM TH in the absence of tyrosine at pH 8.2 and 7.2, 25 °C. The initial velocities obtained (subtracting residual rates of BPH_4 oxidation in the absence of TH) were 0.004 and 0.0002 s^{-1} at pH 8.2 and 7.2, respectively. The former is of a magnitude comparable to the rate of oxidation induced by 1.0 μM TH in the presence of substrate. Thus, TH either catalyzes or initiates the oxidation of BPH_4. One interpretation is there may be transient formation of the TH hydroxylating intermediate in the absence of tyrosine if a common mechanism of oxygen activation is being utilized.

We have demonstrated that PAH can utilize H_2O_2 as a cofactor for the oxidation of tetrahydropterins in the absence of substrate (Dix & Benkovic, 1985; Benkovic et al., 1986). Incubation of 2.0 μM TH with 20 or 200 μM H_2O_2 at pH 8.2 and 7.2 failed to reveal a greater rate of BPH_4 oxidation than obtained in the experiments described above. Since H_2O_2/BH_4 support a hysteretic cycle, oxidation of TH may control the pterin oxidase activity. These conditions were comparable to those previously utilized to establish the H_2O_2-dependent tetrahydropterin oxidase activity of PAH.

Peroxide-Dependent Hydroxylation of Tyrosine by TH. We have speculated (Dix et al., 1985; Dix & Benkovic, 1985; Benkovic et al., 1985) that the most likely structure for the hydroxylating species generated by PAH from tetrahydropterin and O_2 is a peroxytetrahydropterin. This implies that a peroxide might be able to substitute for tetrahydropterin and O_2 as a cofactor for phenylalanine hydroxylation by PAH and, by extension, tyrosine by TH. Data presented in Table IV support this hypothesis. Incubation of TH with a 10-fold excess of H_2O_2 results in a slightly greater than stoichiometric (with respect to TH) hydroxylation of tyrosine. No hydroxylation occurs in the absence of either TH or H_2O_2. Curiously, a 100-fold excess of H_2O_2 to TH also results in no tyrosine hydroxylation. TH activity assays demonstrated that, after incubation with 100-fold excess peroxide, the enzyme is devoid of activity, whereas the 10-fold excess peroxide-treated enzyme still retains about 80% of full activity. Thus, the lack of tyrosine hydroxylation noted in the presence of 100-fold excess H_2O_2 is due to the enzyme being inactivated by peroxide

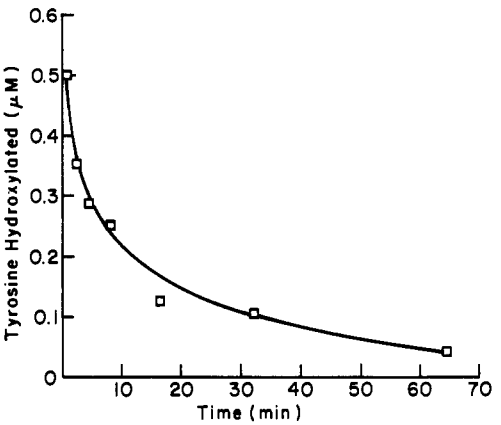


FIGURE 3: Tyrosine hydroxylation by TH in the absence of BPH_4. The experiment was performed in two steps. In step 1, TH (1.0 μM) and BPH_4 (1.0 μM) were mixed in 1.0 mL of 0.02 M Tris, pH 8.2, 25 °C. Reaction was monitored by assays for BPH_4 (see Experimental Procedures) until all BPH_4 had been metabolized (<30 s). In step 2, reactions were quenched by addition of tyrosine (1.0 μCi/μmol) to 20 μM at the given time point, where $t = 0$ is $t = 30$ s from step 1. Tyrosine hydroxylation was determined by release of ^{3}H into ^3H_2O; all values are means of triplicate experiments with standard errors less than 10% for all data points.

at a rate faster than its ability to utilize peroxide as a cofactor for tyrosine hydroxylation. We also attempted to utilize other peroxides as hydroxylation cofactors. Incubation of TH with 10- and 100-fold excess *tert*-butyl hydroperoxide resulted in no tyrosine hydroxylation and complete enzyme inactivation. Incubation with *m*-chloroperoxybenzoic acid results in complete TH inactivation and nonenzymic tyrosine hydroxylation as demonstrated by showing an equal amount of hydroxylation occurring in the absence of TH. Thus, *m*-CPBA is activated sufficiently to transfer oxygen to tyrosine in the absence of a catalyst (e.g., TH). We have also noted that TH slowly self-inactivates during turnover (data not shown), a process previously seen with PAH (Shiman, 1985).

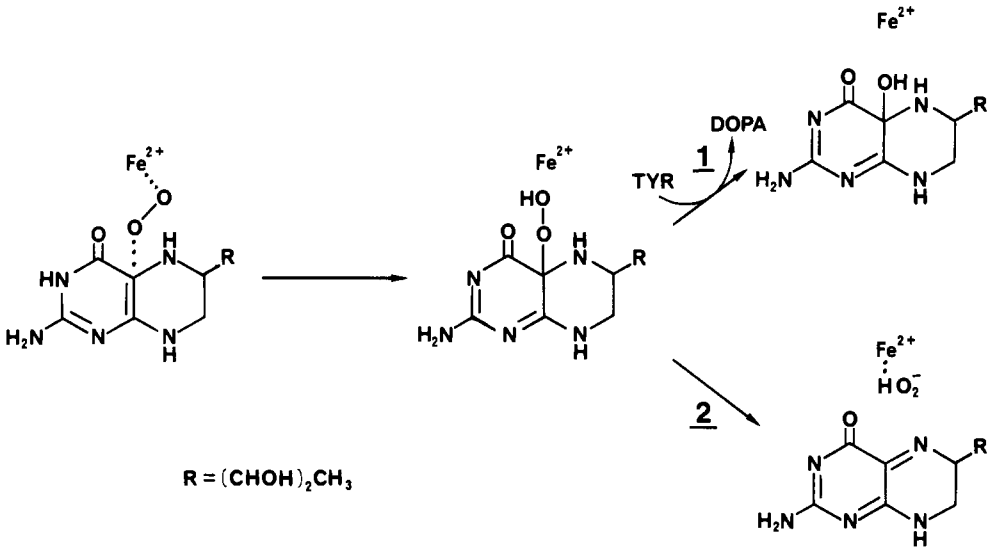
Generation of a Hydroxylating Species by TH in the Absence of Tyrosine. The experiments above suggest the possibility of a transient hydroxylating species being generated by TH from BPH_4 and O_2 which could later be scavenged by tyrosine in a hydroxylation event, as in reactions 1 and 2

$$TH + O_2 + BPH_4 \rightarrow [TH{\cdot}O_2{\cdot}BPH_4] \quad (1)$$

$$[TH{\cdot}O_2{\cdot}BPH_4] + Tyr \rightarrow TH + 4aOH{\cdot}BPH_4 + DOPA \quad (2)$$

($TH{\cdot}O_2{\cdot}BPH_4$ is an unspecified hydroxylating species). Thus, TH (1.0 μM), BPH_4 (1.0 μM), and O_2 were incubated for a given period of time (eq 1) followed by quenching of the reaction with tyrosine (eq 2). Data presented in Figure 3 suggest that the proposed reactions are occurring. When tyrosine is added after 1 min, about 50% (with respect to TH) hydroxylation occurs whereas longer time periods between eq 1 and 2 result in less hydroxylation. This suggests that the intermediate breaks down with time. Further experiments, however, suggested that the intermediate may not contain BPH_4. When the rate of BPH_4 oxidation to BPH_2 was followed under eq 1 conditions, complete oxidation occurred in ca. 30 s. In addition to demonstrating that hydroxylation is not occurring due to the presence of residual BPH_4, this result implies formation of H_2O_2 from BPH_4 and O_2 vs. transient formation of a $TH{\cdot}BPH_4{\cdot}O_2$ hydroxylating species. Subsequent utilization of the generated H_2O_2 by TH for tyrosine hydroxylation, as demonstrated above, could then occur. Inclusion of 15 μg/mL catalase failed to significantly inhibit this

Scheme II



reaction by >10%; thus, if H_2O_2 is generated, it is bound tightly to TH.

Discussion

Our results provide evidence that TH and PAH share common mechanistic elements of catalytic turnover—both enzymes contain Fe^{2+} and convert their tetrahydropterin cofactors to 4a-carbinolamines concomitant with substrate hydroxylation. A reasonable mechanistic hypothesis for the chemistry of TH oxygen activation and hydroxylation is presented in Scheme II. The putative hydroxylating intermediate peroxytetrahydropterin has two possible fates, depending upon whether substrate hydroxylation occurs. The first fate is completion of the TH catalytic cycle by transfer of its outermost oxygen atom into tyrosine, forming DOPA and 4a-carbinolamine. The latter species spontaneously dehydrates, forming BPH_2, the final product of TH catalytic turnover. Alternatively, if substrate hydroxylation cannot or does not occur, TH oxidizes BPH_4 directly to BPH_2. This pathway occurs under nonphysiological reaction conditions—either when there is no tyrosine available to the enzyme to be hydroxylated or when the reactions are run at a nonphysiological pH (8.2 vs. 7.2). This TH activity parallels "uncoupled" PAH turnover (Dix & Benkovic, 1985) that results from the utilization of *p*-chlorophenylalanine as a substrate for the enzyme; an energetically more difficult substrate to hydroxylate. Thus, both TH and PAH catalyze BPH_4 oxidation directly to BPH_2 when substrate hydroxylation does not occur.

It appears reasonable that TH and PAH also utilize their Fe^{2+} centers for generation of the 4a-peroxytetrahydropterin. With both enzymes, the Fe^{2+} center may serve as a site for O_2 binding. In addition, it provides orbital overlap, thereby overcoming the spin-forbidden nature of reacting triplet O_2 with BPH_4 as required for generating a peroxytetrahydropterin. This is an interesting contrast to bacterial aromatic amino acid hydroxylases which utilize reduced flavins for generating hydroxylating intermediates (4a-hydroperoxyflavins; Entsch et al., 1976). These enzymes, which share an obvious mechanistic relationship to tetrahydropterin-dependent hydroxylases, do not contain metal centers (Ballou, 1982). However, reduced flavins react spontaneously with O_2 to form peroxyflavins through a radical recombination mechanism (Bruice, 1984), a pathway that is less accessible to tetrahydropterins owing to the greater instability of their semiquinone form. Consequently, all tetrahydropterin-utilizing enzymes have metal centers, ferrous iron for mammalian PAH (Gottschall et al., 1982), TH (this work), and tryptophan hydroxylase (Kuhn & Lovenberg, 1985), while a PAH from *Chromobacterium violaeceum* contains copper (Pember et al., 1986).

While TH and PAH appear to share certain features of oxygen activation and processing, important differences between the enzymes are revealed by a divergence in the ways in which they interact with peroxides. This is almost certainly a function of a difference in the Fe^{2+} ligand environments between the two enzymes. This difference was initially inferred from the fact that we are able to isolate TH in the ferrous form while PAH is always isolated as ferric and must be reduced to ferrous for catalytic activity (Marota & Shiman, 1984; Wallick et al., 1984). More importantly, PAH rapidly self-inactivates during turnover if not protected by catalase whereas our TH preparation does so only at an extremely slow rate. PAH inactivates in uncoupled turnover most likely owing to its ability to perform Fenton's chemistry on a bound H_2O_2 (derived from the putative 4a-peroxy adduct) thereby generating the extremely reactive hydroxyl radical (Dix & Benkovic, 1985) which probably acts by oxidizing critical amino acid

$$PAH{\cdot}Fe^{2+} + HOOH \rightarrow PAH{\cdot}Fe^{3+} + {}^{-}OH + {\cdot}OH \quad (3)$$

residues to inactivate the enzyme. The above reaction is energetically favorable for PAH, assuming that its Fe center has a reduction potential similar to that of free aqueous iron (Fe^{3+}/Fe^{2+}, +0.4 V NHE, whereas $H_2O_2 + e^{-} \rightarrow {}^{-}OH + {\cdot}OH$ is +0.51 V NHE; Sugimoto & Sawyer, 1984, 1985). For TH, if one invokes a slightly more positive reduction potential, reduction of Fe^{3+} is less favored so that the Fe center can exhibit alternative modes of peroxide activity (see below) in competition with Fenton's chemis These alternative reactivities cannot occur with PAH o the facile nature of eq 3. Treatment of TH with more oxidized peroxides such as *tert*-butyl hydroperoxide or *m* results in enzyme inactivation and an inability to reac these peroxides in any other way.

The unique ability of TH to hydrox e tyrosine utilizing H_2O_2 as a cofactor (as compared to PAH which is unable to hydroxylate phenylalanine by any means other than normal catalytic turnover)[3] thus may be a manifestation of two fa-

[3] T. A. Dix, unpublished data.

vorable kinetic/thermodynamic properties of the system. First, tyrosine is energetically easier to hydroxylate than phenylalanine, due to ring activation toward electrophilic addition provided by its *p*-hydroxyl moiety. Second, the difference in redox potential of the Fe^{2+} center in TH vs. PAH enables H_2O_2 activation in competition with the Fenton chemistry that predominates in the latter system. The required active-site H_2O_2 may be furnished from solution or by generation from the $E{\cdot}BPH_4{\cdot}O_2$ complex (Figure 3). For TH, there are two possible mechanisms by which the Fe^{2+} center activates H_2O_2 for hydroxylation: the first is serving as a Lewis acid for increasing the electrophilicity of the peroxide oxygen transferred into tyrosine, while the second is forming a discrete ferryl species on the enzyme, i.e.

$$Fe^{2+} + H_2O_2 \rightarrow [Fe{=}O]^{2+} + H_2O \qquad (4)$$

which then may be capable of hydroxylating tyrosine. While a ferryl species has never been demonstrated as forming with a non-heme iron enzyme, there is evidence for transient solution formation when ferrous EDTA is reacted with peroxide (Rush & Koppenol, 1986). Tyrosine is an effective scavenger of this species whereas benzoate is not (Rush & Koppenol, 1986); however, whether oxygen atom transfer vs. oxidative processes are occurring in this system is not known. Thus, the exact role of Fe^{2+} in activating H_2O_2 for tyrosine hydroxylation by TH cannot as yet be determined.

The more fundamental question is whether the above chemistry of H_2O_2 activation by TH is relevant to the shared mechanism of tetrahydropterin-dependent oxygen activation by TH and PAH. For our TH preparation, we feel that it probably is not, on the basis of the following considerations. We have demonstrated that the activated ring of tyrosine but not phenylalanine is hydroxylated by *m*-CPBA at physiological pHs in the absence of any catalysis.[3] In addition, Bruice (1984) has shown that 4a-peroxyflavins are facile oxygen donors to a number of nucleophilic acceptors such as I^-, thioxane, and a variety of secondary and tertiary amines, again in the absence of metal or enzymic catalysts. We have calculated that the oxygen-transferring ability of a putative 4a-peroxytetrahydropterin is comparable to that of a 4a-peroxyflavin.[3] Thus, there may be no need to invoke further Fe^{2+} activation of a 4a-peroxytetrahydropterin by TH for tyrosine hydroxylation; the enzyme simply has to catalyze the generation of a peroxytetrahydropterin and provide a passive environment (i.e., bringing the substrate and activated oxygen species together) for hydroxylation to occur in an analogous fashion to the bacterial flavin-dependent hydroxylases (Entsch et al., 1976; Ballou, 1982). As for PAH, the energetic requirements of hydroxylating an unactivated aromatic ring may require participation of the Fe^{2+} center in further activating the putative 4a-peroxytetrahydropterin. In conclusion, it appears that whereas the overall pattern of tetrahydropterin oxygen activation by TH and PAH is similar, subtle differences in the Fe^{2+} ligand field as well as different thermodynamic requirements for performing hydroxylations of their respective substrates predispose each enzyme toward different reactivities with peroxides.

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Infrared and Raman Studies Show That Poly(dA)·Poly(dT) and $d(AAAAATTTTT)_2$ Exhibit a Heteronomous Conformation in Films at 75% Relative Humidity and a B-Type Conformation at High Humidities and in Solution†

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ABSTRACT: The decadeoxynucleotide $d(AAAAATTTTT)_2$ in duplex form and the double-helical polynucleotide poly(dA)·poly(dT) have been studied by Raman and infrared (IR) spectroscopy under a variety of environmental conditions. The IR spectra have been taken of cast films and compared to the IR spectra of the alternating poly(dA-dT), which shows clear B-genus and A-genus vibrational spectra under conditions of high (>92%) and low (75%) relative humidity (RH). From the IR data, it is shown that $d(AAAAATTTTT)_2$ and poly(dA)·poly(dT) adopt a B-genus conformation in films with high water content. When the relative humidity of the film is decreased, the IR spectra reflect a gradual evolution of the geometry of both $d(AAAAATTTTT)_2$ and poly(dA)·poly(dT) into a form intermediate between the B genus and A genus, but the IR spectrum of a pure A genus has not been obtained. In these DNAs at 75% RH, the IR bands of adenosine have the same frequencies as those found in poly(dA-dT) at 75% RH where the local furanose conformation is C3′ endo/anti, but the thymidine frequencies do not resemble those of poly(dA-dT) at 75% RH but rather those of poly(dA-dT) at high humidities. It is concluded that both poly(dA)·poly(dT) and $d(AAAAATTTTT)_2$ adopt a fully heteronomous duplex geometry in cast films at low humidity. For studies in aqueous solution the Raman effect was employed. As a model for the heteronomous conformation in solution, the duplex poly(rA)·poly(dT) was used. Two methods were devised to estimate the amount of heteronomous conformation for $d(AAAAATTTTT)_2$ and poly(dA)·poly(dT) in solution from the intensities of a sugar–phosphate band and an adenine band obtained by a fit of the Raman spectrum to a sum of Lorentzians bands. These measurements indicate that about 30% of the adenine residues were attached to furanose rings with the C3′-endo/anti conformation on the time scale of the Raman effect. This would mean that no more than about 15% of the furanose rings in the duplex are in this conformation. If the conformation of poly(dA)·poly(dT) is fluctuating rapidly in solution, this small amount of C3′-endo ring pucker might be difficult to detect by other techniques such as NMR.

Three main double-helical families are known for DNAs and synthetic oligo- or polynucleotides, the A, B, and Z families characterized by various physical techniques, X-ray single-crystal or fiber diffraction, NMR, vibrational spectroscopy (IR and Raman), and circular dichroism. Recently, many investigations have been undertaken to determine the detailed geometry of DNA of defined base sequence, because sequence-dependent structural variations could serve as recognition signals in the biological functions of the nucleic acids. Although both strands of the double helix in a given DNA conformation have identical geometries in either the A, the B, or the Z form, there is no absolute requirement that both polynucleotide chains in double-stranded DNA helixes should always be conformationally identical. Heteronomous duplex structures with different geometries for each strand have been suggested to exist in DNA–RNA complexes (Zimmerman et al., 1981; Arnott et al., 1986) and proposed in some cases of synthetic polynucleotides with homopurine and homopyrimidine strands (Thomas & Peticolas, 1983; Arnott et al., 1983; Nishimura et al., 1986). The poly(dA)·poly(dT) Raman spectrum recorded in aqueous solution at low temperature has a band that led Thomas and Peticolas to propose that poly(dA)·poly(dT) solutions contain at low temperature a measurable amount of C3′-endo/anti conformation in contrast to the uniform C2′-endo/anti conformation of the classical B double helix; at higher temperature this C3′-endo/anti Raman peak either disappears or becomes very broad (Thomas &

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