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Mechanism of "Uncoupled" Tetrahydropterin Oxidation by Phenylalanine Hydroxylase†

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ABSTRACT: Phenylalanine hydroxylase can catalyze the oxidation of its tetrahydropterin cofactor without concomitant substrate hydroxylation. We now report that this "uncoupled" tetrahydropterin oxidation is mechanistically distinct from normal enzyme turnover. Tetrahydropterins are oxygenated to 4a-carbinolamines only during catalytic events involving substrate hydroxylation. In the absence of hydroxylation tetrahydropterins are oxidized directly to quinonoid dihydropterins. Stoichiometry studies define a ratio of two tetrahydropterins oxidized per O_2 consumed in uncoupled enzyme turnover, thus indicating the complete reduction of O_2 to H_2O. Complementary results establish the lack of H_2O_2 production by the enzyme when uncoupled and define a tetrahydropterin oxidase activity for the enzyme. Thus, the hydroxylating intermediate of phenylalanine hydroxylase may be discharged in two ways, by substrate hydroxylation or by electron abstraction. A mechanism is proposed for the uncoupled oxidation of tetrahydropterins by phenylalanine hydroxylase, and the significance of these findings is discussed.

Oxygen activation by the mammalian mixed function oxidase phenylalanine hydroxylase (PAH)[1] requires a tetrahydropterin and appears to involve O_2 interaction with the ferrous center of the non-heme enzyme (Wallick et al., 1984; Marota & Shiman, 1984). However, the structure of the hydroxylating intermediate formed by PAH and the mechanism for its formation are, at present, unknown. Concurrent

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[1] Abbreviations: PAH, phenylalanine hydroxylase; DHPR, dihydropteridine reductase; 6-MPH_4, 6-methyltetrahydropterin; PH_4, tetrahydropterin; GC–MS, gas chromatography–mass spectrometry; *p*-Cl-Phe, DL-*p*-chlorophenylalanine; Phe, L-phenylalanine; DTT, dithiothreitol; HRP, horseradish peroxidase; PAR, 4-(2-pyridylazo)resorcinol; BHT, 2,6-di-*tert*-butyl-4-methylphenol; Tris, tris(hydroxymethyl)aminomethane; Dopa, L-3,4-dihydroxyphenylalanine.

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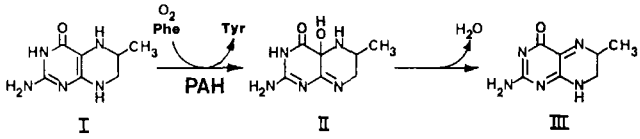
Scheme I



with the hydroxylation of phenylalanine to tyrosine, the tetrahydropterin (structure I in Scheme I) is converted to a 4a-carbinolamine (II) (Lazarus et al., 1981, 1982) which then dehydrates spontaneously to a quinonoid dihydropterin (III). During normal catalytic turnover, PAH utilizes 1 mol of tetrahydropterin per tyrosine. However, in the presence of certain unnatural substrates or cofactors, an "uncoupling" of PAH turnover can occur, resulting in O_2 reduction at the expense of tetrahydropterin in the partial or complete absence of substrate hydroxylation (Storm & Kaufman, 1968; Fisher & Kaufman, 1973). Since this uncoupling presumably represents an alternative mode for discharge of the hydroxylating intermediate, we have studied this process in some detail as a probe for the mechanism of oxygen activation by PAH.

Experimental Procedures

Materials

Rat liver PAH was purified and assayed by the method of Shiman et al. (1979), giving a specific activity of ca. 6 units/mg of protein. This value typically varies with different PAH purifications and is a function of the age of the purified enzyme; hence, it was necessary to determine active PAH concentration immediately prior to each experiment. All reported PAH concentrations and kinetic values are based on active enzyme vs. the total theoretical activity of 12 units/mg (Shiman & Jefferson, 1979). Dihydropteridine reductase (DHPR) was purified and assayed by the method of Craine et al. (1972). 6-Methyltetrahydropterin (6-MPH_4) and tetrahydropterin (PH_4) were prepared as previously described (Storm et al., 1971). All other biochemicals were purchased from standard supply houses at highest available purity. Distilled, deionized water was used in the preparation of all buffers.

Methods

Kinetic assays were performed on a Gilford 252 spectrophotometer. UV spectra were obtained on either a Hewlett-Packard 8450A diode array spectrophotometer or a Varian/Cary 219 double-beam spectrophotometer. Fluorescent measurements were made on a Perkin-Elmer MPF 44A spectrofluorometer. GC–MS was performed on a Finnigan 3200, 9500, and 6000 system. Solution [O_2] was monitored in air-saturated buffers at 25 °C by using a Clark oxygen electrode.

Assay for p-Cl-Phe Purity. DL-*p*-Chlorophenylalanine (*p*-Cl-Phe; Sigma) was assayed by GC–MS for the absence of contamination by phenylalanine (Phe). Samples of *p*-Cl-Phe and Phe were derivatized to *N*(*O*)-trifluoroacetyl *n*-butyl esters (Kaiser et al., 1974) and injected onto a 6 ft 3% OV-17 on Chromosorb WHP 80/100 column. Elution was at standard gas flows by using a temperature gradient of 100–250 °C at 5 °C/min. Diagnostic mass spectra (Kaiser et al., 1974) were obtained for Phe at a retention time of 14.3 min and for *p*-Cl-Phe at 16.0 min. When injected alone, the derivatized *p*-Cl-Phe sample gave no ion current above background at 14.3 min, thus demonstrating the absence of Phe contamination. No other peaks with greater than 1–2% intensity relative to the p-Cl-Phe peak were observed in the total ion profile of this compound.

PAH Turnover Assays. All assays were performed at 25 °C in 1.0 mL of 0.1 M borate, pH 8.4, unless otherwise indicated. PAH, substrate, and any other reaction components were preincubated for 3 min followed by cofactor addition to initiate enzyme turnover. Cofactor oxidation was monitored with time at 334 nm for 6-MPH_4 and at 340 nm for PH_4. ($\Delta\epsilon$ for oxidation to quinonoid dihydropterins is 3200 and 2860 M^{-1} cm^{-1}, respectively, for the two cofactors at the given wavelengths.) Substrate hydroxylation was monitored with time at 275 nm (overall $\Delta\epsilon$ values for Phe and p-Cl-Phe hydroxylation at this wavelength in the presence of DTT oxidation are 1700 and 1850 M^{-1} cm^{-1}, respectively). If dihydropterin recycling systems were required, the incubations also contained either 6.0 mM DTT or 150 μM NADH and 24 μg/mL purified DHPR.

Substrate hydroxylation was also determined by measuring the fluorescence of nitrosonaphthol derivatives (Waalks & Udenfriend, 1957). Standard curves were constructed with tyrosine when Phe was the substrate and 10/1 *m*-chlorotyrosine/tyrosine when p-Cl-Phe was the substrate, in order to properly reflect the product profile of *p*-Cl-Phe hydroxylation by PAH (Guroff et al., 1966). Linear fluorescent responses were obtained from 0–20 nmol of product in both systems, and less than 1 nmol could be accurately determined. The overall fluorescence response of the *m*-chlorotyrosine/tyrosine mixture was only about 40% that obtained when tyrosine was used on an equimolar basis.

4a-Carbinolamine Spectra and Kinetics. Spectra of the 4a-carbinolamines of 6-MPH_4 and PH_4 generated during uncoupled PAH turnover were obtained on the Hewlett-Packard spectrophotometer. A cuvette containing 3.0 μM PAH and 1.0 mM substrate in 0.1 M borate, pH 8.4, at 25 °C served as reference. After addition of 20 μM cofactor to initiate PAH turnover, complete absorbance spectra from 400 to 220 nm were recorded every 5 s and stored for further analysis. When the absorbance at 244 nm had maximized, diagnostic of maximal 4a-carbinolamine formation, the incubation was shown to contain no reduced pterin by the dichlorophenolindophenol assay (Lazarus et al., 1982). The amount of quinonoid dihydropterin at any time was determined by the absorbance at 400 nm (ϵ_{400} for quinonoid 6-MPH_2 and PH_2 are 1875 and 1370 M^{-1} cm^{-1} respectively), as the 4a-carbinolamines have no absorbances at this wavelength (Lazarus et al., 1981). The amount of absorbance in overlapping spectral regions due to the quinonoid dihydropterins was calculated at every 5-nm intervals by using previously determined extinction coefficients and subtracted from the total absorbances, leaving the absorbance spectra of the 4a-carbinolamines. At longer times, each spectrum decayed to one of 20 μM quinonoid dihydropterin, demonstrating the dehydration of the 4a-carbinolamines. Rearrangement of the quinonoid dihydropterins to the more stable 7,8-dihydropterins was demonstrated to be slow on the time scale of the experiment for both cofactors.

Rate constants for the formation (k_1) and decay (k_2) of the 4a-carbinolamines were determined under standard incubation conditions for each tetrahydropterin cofactor. Reaction progress curves for changes in [4a-carbinolamine] were constructed as follows. The λ_{max} in the UV spectrum of each 4a-carbinolamine was monitored with time although it includes absorbance changes of both the 4a-carbinolamine and changes due to overall oxidation of tetrahydropterin to quinonoid dihydropterin. The latter component could be factored out by simultaneously monitoring this conversion at a longer wavelength in which the 4a-carbinolamine does not absorb. The

wavelengths monitored for 6-MPH_4 were 244 and 334 nm, respectively (Lazarus et al., 1983), and for PH_4 were 255 and 340 nm, respectively. The longer wavelengths are isobestic for the quinonoid to 7,8-dihydropterin transition. Plots of ln [4a-carbinolamine] vs. time revealed first-order decays (k_2) which, when extrapolated back to zero time, gave total formation of 4a-carbinolamine in the incubations. Rate constants for 4a-carbinolamine formation (k_1) were determined from plots of the ln of the difference between actual [4a-carbinolamine] and extrapolated [4a-carbinolamine] vs. time at early incubation times where [4a-carbinolamine] was still increasing.

H_2O_2 Assays. [H_2O_2] was determined in two ways: (a) by the method of Zaitsu & Ohkura (1980) using the horseradish peroxidase (HRP)/H_2O_2 dependent oxidation of tyramine to a fluorescent derivative and (b) by the method of Matsubara et al. (1983) utilizing the formation of a titanium/H_2O_2/4-(2-pyridylazo)resorcinol (PAR) mixed ligand complex absorbing at 508 nm. For each method, concentration dependence curves were linear from 0 to 10 nmol of H_2O_2, and less than 1.0 nmol could be accurately determined. Incubations were always assayed after complete cofactor oxidation to avoid any complications due to the presence of tetrahydropterins (see below).

Catechol Scavenging of Ferric PAH. The rate of ferric PAH formation from ferrous PAH during uncoupled turnover was determined as described by Wallick (1985) and R. Shiman.[2] This assay utilizes the ability of catechols to inhibit turning over PAH by binding tightly to the ferric form of the enzyme; thus, the greater rates of inhibition noted with increasing [catechol] can be used to calculate the rate of ferric PAH formation from catalytically active ferrous PAH. Incubations contained 1.0 μM PAH, 1.0 mM *p*-Cl-Phe, 50 μM 6-MPH_4, 6.0 mM DTT, and 24 μg/mL catalase in 0.1 M Tris, pH 8.4 at 25 °C. The rate of hydroxylation was monitored for 3 min at 275 nm prior to the addition of catechol to inhibit enzyme turnover. Tris buffers were utilized for these experiments instead of borate due to the latter's ability to form complexes with *cis*-dihydroxy compounds, thereby rendering catechol incapable of scavenging ferric PAH. Controls demonstrated that the degree of uncoupling observed for a given enzyme sample was similar in Tris and borate buffers, although the overall rate of hydroxylation was slower in Tris (see below).

The tetrahydropterin oxidase activity of PAH was also assayed by this method for the presence of a ferric state during turnover. Incubations contained 1.0 μM PAH, 20 μM 6-MPH_4, and 20 μM H_2O_2 in 0.1 M Tris, pH 8.4 at 25 °C. 6-MPH_4 oxidation was monitored at 334 nm for 1 min prior to the addition of catechol to inhibit enzyme turnover.

Results

Evaluation of Buffers for Uncoupled PAH Turnover. Incubations must be performed at pHs greater than 8 in order to detect the formation and decay of the 4a-carbinolamine intermediate as its dehydration is acid catalyzed (Lazarus et al., 1981). However, Tris inhibits the oxidation of both Phe (Marota & Shiman, 1984) and *p*-Cl-Phe (data not shown) by about 75%, in the latter case slowing down PAH turnover enough so that the observed rate constant for 4a-carbinolamine formation was less than its observed decay constant. This experimental difficulty was overcome by utilizing borate buffer in place of Tris. Second-order rate constants for the oxidation of 20 μM 6-MPH_4 in the presence of 1.0 mM *p*-Cl-Phe in 0.02 M borate, pH 8.4, 0.02 M Tris, pH 8.4, and 0.02 M phosphate, pH 6.8 at 25 °C, were 0.0048, 0.0012, and 0.0009 μM^{-1} s^{-1}, respectively, at 3.0 μM PAH. Since the 4a-carbinolamine was detectable in the borate system, this buffer was utilized for all experiments. It is significant that uncoupled PAH turnover proceeds much more rapidly at pH 8.4 than at 6.8, in direct contrast to coupled turnover (Marota & Shiman, 1984).

Table I: Products of Coupled and Uncoupled PAH Turnover[a]

substrate	cofactor	hydroxylation (μM)	4a-carbinolamine formation (μM)	O_2 consumed (μM)
Phe	6-MPH_4	18.0	18.2	18.5
p-Cl-Phe	6-MPH_4	6.4	6.7	12.6
Phe	PH_4	10.5	10.5	14.3

[a] Incubations contained 0.1 M borate (pH 8.4), PAH (3.0 μM), substrate (1.0 mM), and cofactor (20 μM) in a total volume of 1.0 mL; performed and analyzed as described under Methods.

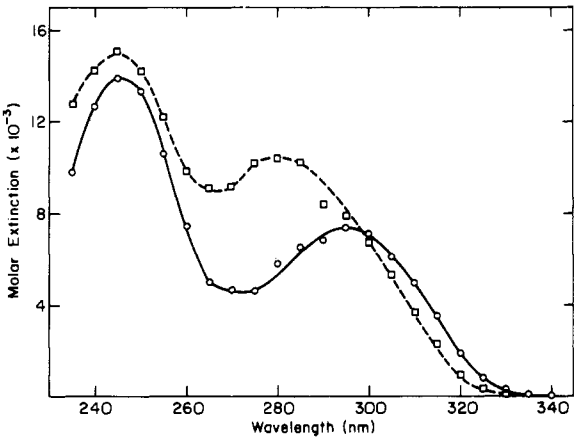


FIGURE 1: UV spectra of 4a-OH-6-MPH_4 (○) and 4a-OH-PH_4 (□) formed during partially uncoupled PAH turnover. Conditions for 4a-carbinolamine generation as described under Experimental Procedures.

Products of Uncoupled PAH Turnover. Tetrahydropterin oxidation was uncoupled from substrate hydroxylation in two ways, first by utilizing an unnatural substrate (*p*-Cl-Phe) and second by utilizing a cofactor known to promote uncoupling (PH_4). In each system (and in a fully coupled control utilizing 6-MPH_4 and Phe), reactions were run under cofactor-limiting conditions and the products of the turnover quantitated to enable a stoichiometric evaluation of reaction characteristics.

Results of these experiments are presented in Table I. In the control reaction, equimolar O_2 consumption, 6-MPH_4 oxidation, and tyrosine formation are noted, characteristic of tightly coupled PAH turnover. Total hydroxylation does not equal total 6-MPH_4 oxidation due to 0.5 mol of 6-MPH_4/mol of PAH being consumed to reduce the ferric center of PAH in an obligatory non-oxygen-consuming activation step.[2,3] In contrast, both modes of uncoupling show an amount of hydroxylation much less than tetrahydropterin oxidation, with the *p*-Cl-Phe/6-MPH_4 system about 65% uncoupled and the Phe/PH_4 system about 44% uncoupled. In addition, Table I also records that less than stoichiometric amounts of O_2 per tetrahydropterin are consumed during both modes of uncoupling and that the amount of O_2 consumed varies inversely with the extent of uncoupling observed.

The 4a-carbinolamine is generated in both uncoupling systems. Figure 1 shows UV spectra of the 4a-carbinolamines of 6-MPH_4 and PH_4, each generated during partially uncou-

[2] R. Shiman, personal communication.

[3] L. Bloom, personal communication.

Table II: H_2O_2 Production during PAH Turnover[a]

system	H_2O_2 (μM)[b] A	B
PAH + 6-MPH_4 + Phe[c]	0.5	0.7
PAH + 6-MPH_4 + *p*-Cl-Phe[c]	1.5	1.2
PAH + PH_4 + Phe[c]	0.4	1.0
PAH + 20 μM H_2O_2[d]	19.4	21.2
6-MPH_4 + 20 μM H_2O_2[d]	19.1	20.0
20 μM H_2O_2[e]	20.1	20.0
PAH + 20 μM H_2O_2[e]	17.5	18.0

[a] All incubations were performed in 0.1 M borate, pH 8.4 at 25 °C. H_2O_2 determined as described under Methods. Concentrations of PAH, substrates, and cofactors are as in Table I. [b] Column A as determined with the tyramine/HRP assay; column B as determined with the Ti/PAR assay. Each experiment was performed in duplicate. [c] Catalytic systems. H_2O_2 determined after a 1.5-min incubation, immediately after oxidation of cofactor was complete. [d] Assayed after a 1.5-min incubation. [e] Assayed after a 20-min incubation.

pled PAH turnover under standard assay conditions. The spectrum of the 4a-carbinolamine of 6-MPH_4 is identical with that already reported (Lazarus et al., 1981). The spectrum of the 4a-carbinolamine of PH_4, not previously reported, shows similar spectral characteristics to the 4a-carbinolamine of 6-MPH_4. Each spectrum decayed in a first-order fashion, enabling determination of the total amount of 4a-carbinolamine formed in each incubation (Lazarus et al., 1983). As seen in Table I, the total formation of 4a-carbinolamine equals the total hydroxylation in each uncoupling system; thus, the 4a-carbinolamine forms only during the coupled portion of PAH turnover.

The rate constants for formation (k_1) and decay (k_2) of the 4a-carbinolamines were determined. The k_1 and k_2 for the p-Cl-Phe/6-MPH_4 system were 0.0048 μM^{-1} s^{-1} and 0.045 s^{-1}, respectively, and were 0.0075 μM^{-1} s^{-1} and 0.027 s^{-1}, respectively, for the Phe/PH_4 system with each system at 3.0 μM PAH. The 4a-carbinolamine of PH_4 thus appears to be more stable than that of the 4a-carbinolamine of 6-MPH_4 in borate buffer. Lazarus et al. (1981) reported a k_2 for 6-MPH_4 in 0.01 M Tris, pH 8.45, of 0.008 s^{-1}; thus, borate may catalyze dehydration of the 4a-carbinolamine. The k_1 for the p-Cl-Phe/6-MPH_4 system was also evaluated by measuring the rate of hydroxylation. The value obtained (0.0054 μM^{-1} s^{-1}) confirms that the 4a-carbinolamine forms concurrent with substrate hydroxylation, as previously demonstrated for fully coupled PAH turnover (Lazarus et al., 1981).

The values recorded in Table I and the kinetic constants above are average values derived from at least eight separate experiments. Different preparations of PAH varied up to 30%; however, a given PAH preparation was internally consistent (standard deviations <5%). The presence or absence of catalase in the incubation mixtures did not affect any of the stoichiometric values.

H_2O_2 Production during PAH Turnover. A logical product of uncoupled PAH oxidation of tetrahydropterin is H_2O_2. We therefore assayed standard incubation mixtures for the presence of H_2O_2 immediately after completion of tetrahydropterin oxidation. As shown in Table II, only trace amounts of H_2O_2 were detected in the coupled and uncoupled PAH systems, whereas added H_2O_2 could be detected quite readily. This is contrary to the results of Storm & Kaufman (1968), who provided indirect evidence of H_2O_2 production during *p*-Cl-Phe hydroxylation by PAH. They assayed PAH turnover by coupling NADH oxidation to dihydropterin reduction with DHPR and demonstrated a greater rate of NADH oxidation when HRP was included in the incubations. The extra NADH oxidation was ostensibly due to the utilization of additional

Table III: NADH Oxidation by DHPR during PAH Turnover–HRP Effect[a]

system	initial velocity of NADH oxidation (μM/min)
PAH + 6-MPH_4 + *p*-Cl-Phe	0.79
PAH + 6-MPH_4 + *p*-Cl-Phe + HRP	1.85
6-MPH_4 + *p*-Cl-Phe + HRP	1.61

[a] All reactions run in 0.1 M borate, pH 8.4 at 25 °C, containing 24 μg/mL DHPR and 150 μM NADH. Initial velocity of NADH oxidation determined by monitoring A_{340} with time [$\Delta\epsilon_{340}$(NADH oxidation) = 6000 M^{-1} cm^{-1}]. Concentration of other reaction components: PAH (1.0 μM), 6-MPH_4 (10 μM), *p*-Cl-Phe (1.0 mM), and HRP 2.6 μg/mL. Each experiment was performed in duplicate.

Table IV: Retention of Enzyme Activity after Uncoupled and Coupled PAH Turnover

conditions[a]	% activity
Phe + catalase	63
Phe − catalase	28
p-Cl-Phe + catalase	42
p-Cl-Phe − catalase	14

[a] All incubations contained 1.0 μM PAH, 1.0 mM substrate, and, if indicated, 24 μg/mL catalase in 0.02 M borate, pH 8.4 at 25 °C; 20 μM 6-MPH_4 was added to initiate emzyme turnover and the absorbance at 334 nm monitored with time until cofactor oxidation was complete. At this time, 100 μL of the incubation mixture was added to a standard assay of 1.0 mM Phe, 50 μM 6-MPH_4, 6.0 mM DTT, and 24 μg/mL catalase in 0.1 M phosphate, pH 6.8 at 25 °C, and the remaining activity of PAH was determined by monitoring at 275 nm, as described under Methods. The control incubation (100% activity) utilized 0.1 μM fresh PAH. Each experiment was performed in duplicate.

6-MPH_4 by HRP as a cofactor for H_2O_2 reduction. However, a HRP oxidation of tetrahydropterins to quinonoid dihydropterins utilizing only trace amounts of H_2O_2 has recently been observed (Armarego et al., 1983). As seen in Table III, HRP promotes a rate of 6-MPH_4 oxidation double that seen with PAH when each is run under the conditions of Storm and Kaufman and assayed by the oxidation of NADH by DHPR. The 6-MPH_4 oxidation by HRP is not NADH/DHPR dependent as 2.6 μg/mL HRP will completely oxidize 20 μM 6-MPH_4 to quinonoid 6-MPH_2 with an observed rate constant of ca. 0.05 s^{-1}. The exact nature of this oxidation is obscure, although the most likely mechanism is a H_2O_2/HRP-*initiated* autoxidation of the tetrahydropterin (Blair & Pearson, 1973; Mager, 1979). We could inhibit the rate of HRP-dependent oxidation of 6-MPH_4 by greater than 90% by adding either 10 μM of the free radical scavenger BHT or 24 μg/mL catalase to the incubation prior to HRP addition.

Retention of Activity in Uncoupled PAH Turnover: Effect of Catalase. The PAH assay mixture must include catalase to maintain full enzyme activity (Kaufman, 1962). However, the lack of significant H_2O_2 production in both coupled and uncoupled PAH turnover raises the question as to the nature of the protection afforded by catalase. Consequently, turnover reactions were assayed for retention of activity in both the absence and presence of catalase (Table IV). It can be seen that catalase protects PAH during both coupled and uncoupled turnover; however, less protection is noted with the uncoupled enzyme. These data imply that in uncoupled vs. coupled turnover either H_2O_2 is being metabolized by PAH or a different mechanism of enzyme inactivation is operative, as similiar amounts of H_2O_2 are formed in both systems (Table II).

Characterization of a PAH Tetrahydropterin Oxidase Activity. The key to the uncoupled oxidation of tetrahydropterin by PAH resides in the less than stoichiometric amount of O_2 consumption observed in both uncoupling systems (Table

Table V: Stoichiometry[a] of 6-MPH_4 Oxidized per O_2 Consumed in Uncoupled PAH Turnover

	total 6-MPH_4 oxidized (μM)	total O_2 consumption (μM)
(1) overall	20.0	12.6
(2) PAH prereduction	−1.5/18.5	0/12.6
(3) hydroxylation	−6.4/12.1	−6.4/6.2
(4) H_2O_2 production	−1.0/11.1	−1.0/5.2

[a] Stoichiometry: 11.1 6-MPH_{4ox}/5.2 O_2 = 2.1.

Table VI: Effect of Changing [H_2O_2] on the Tetrahydropterin Oxidase Activity of PAH[a]

H_2O_2 (μM)	initial velocity[b] of 6-MPH_4 oxidation (μM/min)	total % 6-MPH_4 oxidized[c]	% remaining PAH activity
0	(0)	(0)	(100)
20	6.2	100	60
40	8.1	100	22
80	7.1	59	0
160	4.7	30	0
320	3.2	24	0

[a] All incubations contained 1.0 μM PAH and 20 μM 6-MPH_4 in 0.1 M borate, pH 8.4 at 25 °C. Oxidations initiated by the addition of H_2O_2 after a 1-min preincubation. Each experiment was performed in duplicate. [b] Rate or extent of 6-MPH_4 autoxidation in the presence of PAH subtracted for each experiment. [c] Assayed as described in Table IV.

I). When the coupled portion of the *p*-Cl-Phe/6-MPH_4 system is factored out, it can be demonstrated that 2.1 tetrahydropterins are oxidized per O_2 consumed in uncoupled PAH turnover (Table V). A similiar calculation yields a ratio of 2.0 tetrahydropterins oxidized per O_2 consumed in the uncoupled portion of the Phe/PH_4 system. This implicates O_2 reduction by four electrons to H_2O in uncoupled PAH turnover.

PAH exhibits an oxidase activity toward 6-MPH_4. As seen in Figure 2, when 1.0 μM PAH is incubated with 20 μM 6-MPH_4, a slow tetrahydropterin autoxidation to quinonoid dihydropterin can be monitored at 334 nm. However, when 20 μM of H_2O_2 is added, a second-order 6-MPH_4 oxidation (0.0003 μM^{-1} s^{-1}) at 1.0 μM PAH is noted. No 4a-carbinolamine forms in this oxidation since the formation and decay of this species cannot be detected at 244 nm (cf. Figure 1) even when PAH levels of 6.0 μM are utilized. The oxidation is PAH dependent as omission of the enzyme results in only 6-MPH_4 autoxidation in the presence of H_2O_2. In addition, inclusion of less than stoichiometric amounts of H_2O_2 with PAH results in only an equivalent amount of 6-MPH_4 being oxidized at the faster rate. Finally, the oxidation can also be elicited by the peracid *m*-CPBA (Figure 2). As with H_2O_2, oxidation is directly to quinonoid dihydropterin; however, a faster rate of oxidation is noted (0.0035 μM^{-1} s^{-1}).

Marota & Shiman (1984) have characterized an inactivation of ferrous PAH by H_2O_2. To assess whether this process was occurring during the oxidase activity of PAH, we incubated 1.0 μM PAH and 20 μM 6-MPH_4 with increasing amounts of H_2O_2 and determined the amount of enzyme activity remaining after complete tetrahydropterin oxidation. As

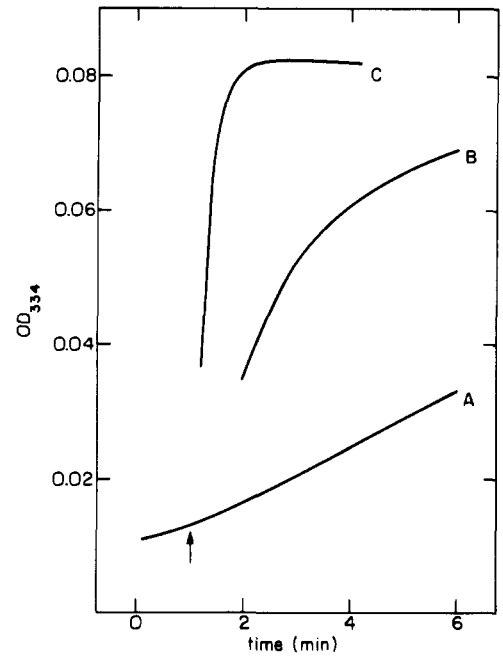
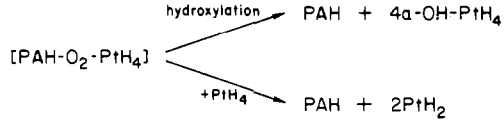


FIGURE 2: Reaction progress curves for the oxidation of 6-MPH_4 by PAH and peroxides. Curve A monitored 1.0 μM PAH and 20 μM 6-MPH_4 in 0.1 M borate, pH 8.4 at 25 °C. Curve B as in (A) except 20 μM H_2O_2 added after 1 min. Curve C as in (A) except 20 μM m-CPBA added after 1 min.

seen in Table VI, oxidation of 6-MPH_4 by an equivalent amount of H_2O_2 results in retention of 60% of the initial PAH activity. However, incubation with larger amounts of H_2O_2 effects complete PAH destruction; i.e., the enzyme cannot be reactivated. The decreases in the initial velocity and extent of 6-MPH_4 oxidation observed with increasing H_2O_2 suggest that enzyme destruction is occurring competitively with 6-MPH_4 oxidation. Even under optimal conditions of turnover (equimolar 6-MPH_4 and peroxide), PAH inactivates at a rate of 1 enzyme per 50 turnovers. Thus, H_2O_2 is deleterious to PAH, and tetrahydropterins "protect" PAH from peroxide-dependent inactivation, presumably by scavinging active oxygen species destructive to the enzyme.

Effect of Changing [Tetrahydropterin] on the Degree of Uncoupling by PAH. The lack of 4a-carbinolamine formation in the uncoupled portion of PAH turnover suggests that a partitioning is occurring between the two tetrahydropterin oxidizing pathways:



This scheme implies that the degree of uncoupling should be first order in [tetrahydropterin]. This was evaluated by varying [6-MPH_4] in uncoupled *p*-Cl-Phe oxidation by PAH. As shown in Table VII, the degree of uncoupling is independent of [6-MPH_4] as parallel changes are seen in the amount of hydroxylation, 4a-carbinolamine formation, and O_2 con-

Table VII: Effect of Changing [6-MPH_4] on the Degree of Uncoupling during PAH Turnover[a]

6-MPH_4 (μM)	hydroxylation (μM)	4a-carbinolamine formation (μM)	H_2O_2 production (μM)	O_2 consumption (μM)	6-MPH_4/O_2 consumed[b]
10	4.0	3.6	0.5	6.5	2.00
20	8.0	8.0	1.0	14.4	1.75
40	16.0	15.4	1.9	27.8	1.76

[a] Incubations performed under the conditions of Table I and analyzed as described under Methods. Each experiment was performed in duplicate. [b] Stoichiometry of 6-MPH_4 oxidized per O_2 consumed in uncoupled PAH turnover. Calculated as in Table V.

sumption when [6-MPH_4] is varied. A similiar experiment utilizing a 10-fold range of [6-MPH_4] produced equivalent results.

Ferric PAH Formation Induced by Uncoupled PAH Turnover. PAH must be stoichiometrically reduced by its tetrahydropterin cofactor (Marota & Shiman, 1984; Wallick et al., 1984) or an alternative electron source (Wallick et al., 1984) to be catalytically active. The likely site of reduction is the iron center, as addition of reducing equivalents results in trapping by the ferrous chelator *o*-phenanthroline (Wallick et al., 1984), a lack of inhibition by the ferric chelator Dopa (Marota & Shiman, 1984), and EPR changes consistent with a ferric to ferrous transition.[4] After reduction, fully coupled, catalytically active PAH reverts to its inactive, oxidized form at a rate that is slow relative to turnover, but measurable (Wallick, 1985).[2] This can be measured by utilizing the ability of catechols such as Dopa to inhibit PAH by binding to its ferric form. At infinite [catechol] the rate of inactivation (k_i) is independent of the catechol trap and thus measures the rate at which turning over PAH reverts to its oxidized form (k_{ox}). Figure 3a plots the ln (hydroxylation rate) vs. time for uncoupled PAH turnover in the absence and presence of various catechol concentrations. As [catechol] is increased, the rate of inactivation increases. The k_i's, determined from the slope of each line in Figure 3a, are replotted vs. [catechol] as double reciprocals in Figure 3b, enabling the determination of k_i at infinite [catechol], or k_{ox}. The k_{ox} thus obtained, 5 μM^{-1} min^{-1}, compares with the initial rate of hydroxylation of 7.6 μM product μM^{-1} min^{-1}, indicating that the enzyme reverts to its ferric state once every 1.5 turnovers when uncoupled. Controls performed under identical conditions but utilizing Phe as the substrate demonstrated that coupled PAH turnover results in only one reversion to the ferric state every 200 turnovers. A figure of 1 reversion per 140 turnovers at pH 6.8 has been independently determined by R. Shiman.[2] These data imply that tetrahydropterin oxidation by PAH in the absence of substrate hydroxylation proceeds primarily or completely through the ferric state of the enzyme, which must then be rereduced by cofactor to continue turnover.

The tetrahydropterin oxidase activity of PAH in the presence of H_2O_2 was also assayed for the rate of reversion to a ferric state during turnover. It could be demonstrated that the enzyme reverts to a ferric state about once per each 6-MPH_4 oxidized, further suggesting a mechanistic similarity between this activity and the uncoupled oxidation of tetrahydropterins by PAH. The difference between this result and that in Table VI suggests that conversion of ferrous PAH to the ferric state is not necessarily coupled to an irreversible inactivation of the enzyme.

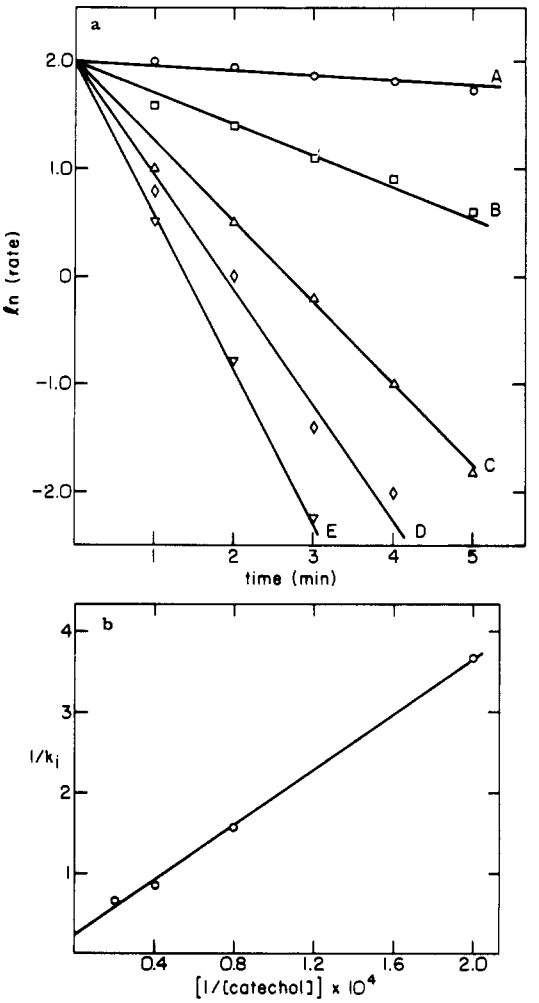


FIGURE 3: Determination of k_{ox} for uncoupled PAH turnover. Incubation conditions as described under Methods; analysis as described under Results. Part a plots the ln (hydroxylation rate) vs. time in the absence and presence of different catechol concentrations. [Catechol] is 0 (A), 50 (B), 125 (C), 250 (D), and 500 μM (E). Each point is the average of three experiments. Part b plots the k_i (determined from the slopes of the lines in part a) vs. [catechol] in a double-recriprocal plot, enabling the determination of k_{ox} (k_i at infinite [catechol]).

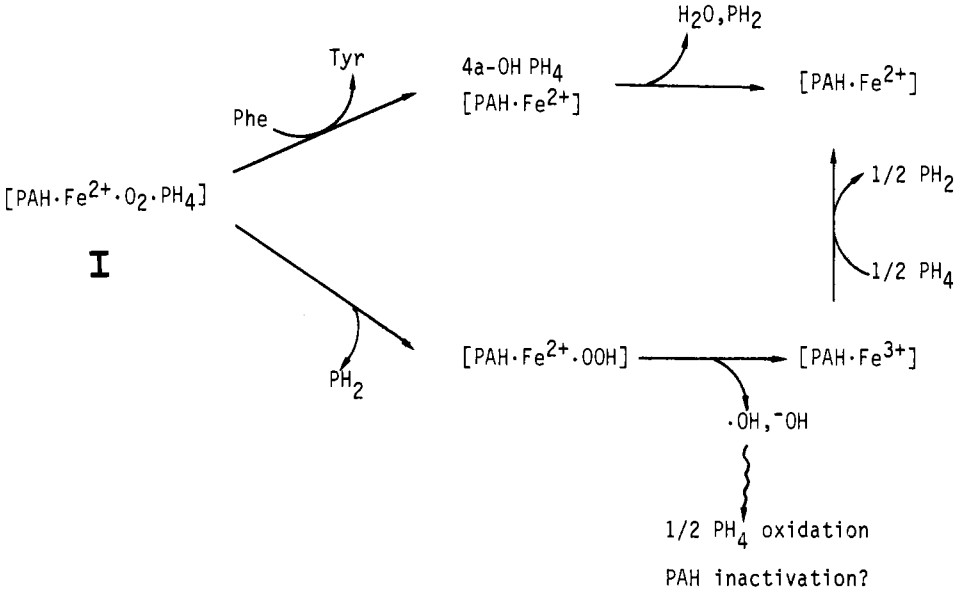
DISCUSSION

We have defined two different pathways for the oxidation of tetrahydropterins during PAH turnover, depending upon whether the oxidation is coupled to or uncoupled from substrate hydroxylation. In the coupled pathway, tetrahydropterins are oxygenated to 4a-carbinolamines concurrent with substrate hydroxylation. Since the hydroxyl group of the 4a-carbinolamine is derived from O_2 (Dix et al., 1985), this species is a residue of the hydroxylating intermediate of PAH. In contrast, uncoupled PAH turnover results in the oxidation of tetrahydropterins directly to quinonoid dihydropterins without the intermediacy of 4a-carbinolamines. Furthermore, the stoichiometry of tetrahydropterins oxidized per O_2 consumed in uncoupled PAH turnover implicates the four-electron reduction of O_2 to H_2O, correlating with the lack of significant levels of H_2O_2 production in the incubations. These general characteristics are observed whether tetrahydropterin oxidation by PAH is uncoupled due to the presence of an unnatural substrate (*p*-Cl-Phe) or cofactor (PH_4). Finally, we have demonstrated the PAH-dependent oxidation of tetrahydropterins by H_2O_2, a reaction that mimics the oxidation of tetrahydropterins during uncoupled PAH turnover.

A mechanistic proposal consistent with all of the above results is presented in Scheme II, as shown for the Phe/PH_4 uncoupling system. Generation of the hydroxylating intermediate (I) requires the binding of substrate, tetrahydropterin, and O_2 to ferrous PAH. This species discharges either through substrate hydroxylation concurrent with tetrahydropterin oxygenation to 4a-carbinolamine or by electron abstraction from tetrahydropterin forming quinonoid dihydropterin and a ferrous dioxygen complex in which oxygen is formally at the level of peroxide. Our postulation of an intermediate ferrous–peroxide complex during uncoupled PAH turnover is based upon two experimental results. First, a change in the partitioning between coupled and uncoupled pathways cannot be effected by changing the concentration of tetrahydropterin

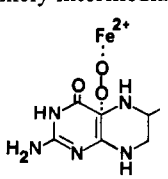
[4] L. Bloom, B. J. Gaffney, and S. Benkovic, unpublished results.

Scheme II



available to the enzyme. This implies that a second mole of tetrahydropterin does not compete with substrate to discharge the enzyme–O_2–tetrahydropterin hydroxylating species; rather, a chemical change involving this species occurs instead of substrate hydroxylation. Second, we are able to show that ferrous PAH, when incubated with peroxide, oxidizes tetrahydropterins to quinonoid dihydropterins. This activity of PAH is a good model for uncoupled tetrahydropterin oxidation because each activity oxidizes 1 mol of tetrahydropterin per peroxide, does not require O_2, and proceeds through the ferric state of the enzyme. The most likely mechanism for this activity is based on Fenton's chemistry (Walling, 1975), as diagrammed in Scheme II. One-electron reduction of the peroxide by ferrous PAH produces ferric enzyme, a hydroxyl radical, and a hydroxyl anion (H_2O). To resume turnover, ferric PAH must be rereduced by another 0.5 mol of tetrahydropterin whereas the hydroxyl radical, a powerful oxidizing agent, also oxidizes another 0.5 mol of tetrahydropterin. Although the exact nature of this reaction remains obscure, Blair & Pearson (1975) have demonstrated that tetrahydropterins are oxidized to quinonoid dihydropterins by Fe^{2+} and H_2O_2 in a complex reaction involving hydroxyl radicals. The other reasonable mechanistic alternative for this activity, formation of a peroxidase-like $[Fe{=}O]^{4+}$ intermediate, appears less likely due to the formation of ferric PAH during turnover and the lack of ligand stabilization for a high-energy iron–oxygen species presumed available in this non-heme enzyme.[5] Non-heme $[Fe{=}O]^{4+}$ species have been observed in nonaqueous media (Sagimoto & Snyder, 1984).

We recently proposed a Fe^{2+}–O_2–tetrahydropterin μ-oxo bridged structure as a likely intermediate in the activation of



oxygen by PAH (Benkovic et al., 1985; Dix et al., 1985). We suggested that hydroxylation of Phe could occur either directly from this species or after rearrangement to a peroxypterin with a structure analogous to peroxyflavins proposed as intermediates in bacterial aromatic amino acid hydroxylase turnover (Entsch et al., 1976). Formation of a peroxypterin involves a shift of electron density into ferrous–dioxygen from tetrahydropterin; we envision uncoupling occurring by a complete transfer of electrons to form quinonoid dihydropterin and the putative ferrous–peroxide species discussed above. This must occur as a result of a less favored orientation between substrate, dioxygen, and cofactor since uncoupling can be induced in the presence of Phe by the use of tetrahydropterin as the cofactor as well as when the energetically less hydroxylatable substrate, *p*-Cl-Phe, is utilized. Different protein conformations (and presumably active site configurations) are established for PAH under a variety of experimental conditions (Shiman et al., 1979; Abita et al., 1984).

Our demonstration of a lack of significant H_2O_2 production during both uncoupled and coupled PAH turnover has several important ramifications. Catalase has long been utilized in assays to protect PAH from inactivation during turnover (Kaufman, 1962) presumably due to its ability to scavenge peroxide released by PAH. However, Webber et al. (1980) have demonstrated that a 50-fold smaller concentration of superoxide dismutase protects PAH equally well as catalase, and they suggest that the protection afforded by catalase is due to a contaminating superoxide dismutase activity. Recent evidence in this laboratory indicates the release of superoxide from PAH during enzyme turnover.[6] The small amount of peroxide detected in both uncoupled and coupled PAH turnover could result from either dismutation of this superoxide or tetrahydropterin autoxidation (Blair & Pearson, 1973; Mager, 1980). The autoxidation may also be a source of superoxide and thus contribute to PAH inactivation (Blair & Pearson, 1973). The lack of *greater* peroxide formation in uncoupled vs. coupled PAH turnover and the greater amount of enzyme inactivation seen in uncoupled turnover in both the absence and presence of catalase require that a different toxic oxygen species be forming in uncoupled PAH turnover. We propose that hydroxyl radicals, formed by breakdown of the ferrous–peroxide complex as discussed above, are these toxic oxygen species. In the absence of scavenging tetrahydropterins, these radicals may oxidize critical amino acid residues on

[5] Recent evidence, however, implicates histidines as ligands to the iron center of PAH (S. Pember and S. Benkovic, unpublished results).

[6] D. Wallick and S. Benkovic, unpublished results.

PAH, thereby rendering the enzyme inactive. This hypothesis can be extended to the use of *p*-Cl-Phe to induce a phenylketonuric state in laboratory animals (Blau, 1979). It has been suggested (Goodwin, 1979) that the irreversible loss of PAH after *p*-Cl-Phe administration may be due to peroxide-dependent inactivation. More specifically, hydroxyl radicals, formed as described above, may be the actual agents of PAH inactivation. The widespread, rapid, loss of PAH activity when rats are administered *p*-Cl-Phe followed by slow recovery of activity as protein synthesis occurs and presumably the compound is cleared from the organism (Ayling & Helfand, 1974) supports this hypothesis, as does the isolation from *p*-Cl-Phe-treated rats of an electrophoretically altered protein (Gâl & Millard, 1971).

In conclusion, we have characterized uncoupled turnover of PAH and have achieved some insights into the chemistry of the hydroxylating intermediate formed by this enzyme. Further work along these lines is in progress.

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Registry No. PAH, 9029-73-6; 6-MPH$_4$, 942-41-6; PH$_4$, 1008-35-1; *p*-Cl-Phe, 7424-00-2.

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