Stoichiometric Reduction of Phenylalanine Hydroxylase by Its Cofactor: A Requirement for Enzymatic Activity†

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ABSTRACT: We have found that rat liver phenylalanine hydroxylase oxidizes a stoichiometric amount of its cofactor, 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$), in a reaction that is independent of phenylalanine. The reaction requires oxygen, and one $6MPH_4$ is oxidized per subunit of enzyme. A quinonoid dihydropterin is directly produced in the reaction, and there is no evidence for the intermediate formation of a 4a-hydroxydihydropterin. Neither hydrogen peroxide nor superoxide anions were detected as products of the oxidation, and phenylalanine hydroxylase appears to be the sole electron acceptor from $6MPH_4$. Therefore, in a functional sense, phenylalanine hydroxylase is reduced by its cofactor. The reduced state of the enzyme is stable to activation by phenylalanine and during catalytic turnover, and the electrons on the reduced enzyme cannot be directly used to drive phenylalanine hydroxylation. Of greatest importance, enzyme reduction appears to be required for the formation of a catalytically active enzyme species. Phenylalanine hydroxylase is chemically and physically altered by reduction. Reduced enzyme exhibits (1) a greatly increased fluorescence, which is quantitatively related to the extent of reduction, (2) an altered UV–visible absorbance spectrum, (3) a greatly increased sensitivity to inactivation by hydrogen peroxide, and (4) a greatly decreased sensitivity to inhibition by Dopa which quantitatively correlates with the increase in enzyme fluorescence. Second-order rate constants, k_r, for the reduction of the enzyme by $6MPH_4$ have been determined and found to vary with pH, temperature, buffer, and enzyme activation: at pH 6.8, 25 °C, and in phosphate buffer, for phenylalanine-activated enzyme $k_r = 15 \times 10^6$ min^{-1} M^{-1}. Tris is a competitive inhibitor with respect to $6MPH_4$ of enzyme reduction and also of catalysis.

Mammalian phenylalanine hydroxylase catalyzes the formation of tyrosine from phenylalanine, molecular oxygen, and a tetrahydropterin cofactor. Only three mammalian monooxygenase are known to require an unconjugated tetrahydropterin for activity (Kaufman & Fisher, 1974), and in all cases, the electrons from the cofactor are used in the reaction to reduce one oxygen atom to water. The mechanism of the catalytic reaction and the role played by the enzyme in this electron transfer are unknown. In attempting to gain more information about this process, we discovered that a stoichiometric amount of tetrahydropterin can be oxidized by the enzyme: the reaction requires oxygen, is independent of phenylalanine, and yields an altered (reduced) enzyme. This reduction appears to be a required step in the formation of catalytically active enzyme. Such a requirement has not been reported before for a tetrahydropterin-dependent hydroxylase. This paper describes these findings.

Materials and Methods

Beef liver catalase and horseradish peroxidase were purchased from Worthington Biochemical Corp., phenylalanine, quinonoid dihydropteridine reductase, bovine blood superoxide dismutase, NADH, and 3,4-dihydroxy-L-phenylalanine were from Sigma Chemical Co., dithiothreitol was from Calbiochemical Corp., and tyramine hydrochloride was from Eastman Kodak Co. 6-Methyltetrahydropterin (Storm et al., 1971) and 6-methyl-5-deazatetrahydropterin (Moad et al., 1979) were prepared, and 6-methyltetrahydropterin solutions were standardized at 266 nm (Shiman et al., 1971). Bio-Gel P4 (200–400 mesh) was from Bio-Rad Corp., and Sephadex G-25 (fine) was from Pharmacia. [^{14}C]Phenylalanine (Amersham/Searle) was purified (Shiman, 1980). Hydrogen peroxide (Fisher) was standardized at 240 nm (Beers & Sizer, 1952).

Fluorescence Measurements. Fluorescence determinations were made with an SLM 8000 fluorometer. All reactions were performed in a quartz cuvette with continuous stirring. For experiments at reduced oxygen tension, the cuvette was sealed with a serum cap, and a continuous flow of argon was maintained over the sample.

UV Measurements. UV and visible spectra were obtained on a Cary 219 scanning spectrophotometer.

Phenylalanine Hydroxylase Preparation and Assays. Rat liver phenylalanine hydroxylase was prepared through step IID by the method of Shiman et al. (1979), and, unless otherwise indicated, activity was assayed as described (Shiman & Gray, 1980). In almost all experiments, iron-treated (Shiman & Jefferson, 1982) enzyme was employed. Protein was determined by the method of Peterson (1977) using crystalline bovine serum albumin as standard.

Dihydropterin Reductase Deglycerination. Dihydropterin reductase (0.4 mL) in 60% glycerin was applied to a 7.8 × 0.8 cm column of Sephadex G-25 (fine) equilibrated and then eluted with 0.01 M potassium phosphate, pH 6.8. Protein-containing fractions were pooled and stored frozen until use. All manipulations were performed at 4 °C.

$6MPH_4$[1] Oxidation. Oxidation of tetrahydropterin was followed by linking the reaction to NADH oxidation with dihydropterin reductase (Craine et al., 1972); the reaction was then measured as a decrease in fluorescence at 460 nm (340-nm excitation) (Udenfriend, 1962). The reactions contained, in a total volume of 1.7 mL of buffer, 10 μM NADH, deglycerinated dihydropterin reductase in excess, and 110–220 μg of phenylalanine hydroxylase. The oxidation was initiated with the addition of $6MPH_4$. Blank rates of oxidation were determined with phenylalanine hydroxylase omitted. For experiments at reduced oxygen tension, the buffer and samples

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[1] Abbreviations: $6MPH_4$, 6-methyltetrahydropterin; Dopa, 3,4-dihydroxy-L-phenylalanine; $7,8\text{-}6MPH_2$, 7,8-dihydro-6-methyldihydropterin; Tris, tris(hydroxymethyl)aminomethane.

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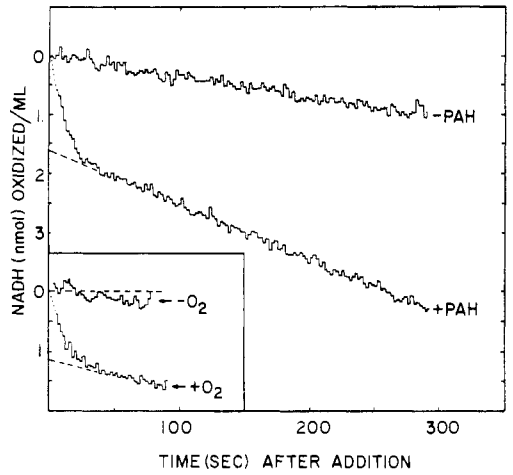


FIGURE 1: $6MPH_4$ oxidation by phenylalanine hydroxylase. "+PAH": time course of NADH oxidation initiated by addition of 2.0 μM $6MPH_4$ to a solution containing 0.1 M potassium phosphate, pH 6.8, 1.9 μM phenylalanine hydroxylase, 10 μM NADH, and excess dihydropteridine reductase. "–PAH": oxidation in a completed reaction mixture missing only phenylalanine hydroxylase. The dotted and dashed lines are extrapolations to zero time. (Inset) "$-O_2$" and "$+O_2$": time courses of NADH oxidation at reduced and normal oxygen tensions, respectively, with 2.1 μM phenylalanine hydroxylase and 2.2 μM $6MPH_4$. The dashed line at the top of the inset represents oxidation in the absence of phenylalanine hydroxylase; in this latter case, the tracing was omitted for clarity. NADH oxidation was monitored as a decrease in fluorescence (Udenfriend, 1962) (see Materials and Methods); the data are presented as nanomoles of NADH oxidized per milliliter of reaction mixture.

were degassed under vacuum and stored under an argon atmosphere. The oxidation of $6MPH_4$ was also monitored as the increase in absorbance at 334 nm (Kaufman, 1971) with the Cary 219 spectrophotometer fitted with a thermostated cell holder. In these latter determinations, the stoichiometry was calculated from the sum of the change in absorbance due to formation of quinonoid dihydropterin (ΔA_{334} = 3900 cm^{-1}) and formation of reduced enzyme (ΔA_{334} = −1600 cm^{-1}).

Calculations for Figure 8. The rate of tyrosine formation for initially unreduced phenylalanine hydroxylase (Figure 8, solid line) was calculated at successive 1-s intervals from the following formula:

$$(\mathrm{Tyr})_t = \sum_0^t \left[V\left(\frac{E_t^*}{E_0}\right)\left(\frac{P_t}{P_0}\right) \right]$$

$(Tyr)_t$ is the amount of tyrosine present at time t; V is the velocity of catalysis of fully reduced enzyme at 1 μM $6MPH_4$, calculated from the fully reduced control reaction (Figure 8, dashed line); E_t^* and P_t are the concentrations of reduced enzyme and $6MPH_4$, respectively, at time t; E_0 and P_0 are the total enzyme and initial $6MPH_4$ concentrations, respectively. For the first 75 s of the reaction

$$\frac{E_t^*}{E_0} = 1 - e^{-tk_r}$$

where k_r is the rate constant of reduction derived in a parallel identical reaction from the rate of change of fluorescence.

At all times, $P_0 = P_t + (\mathrm{Tyr})_{t-1} + E_t^*$.

Results

Stoichiometric Oxidation of $6MPH_4$. Figure 1 shows the effect of adding, in the absence of phenylalanine, 6-methyltetrahydropterin ($6MPH_4$) to a solution containing phenylalanine hydroxylase. In the example shown, NADH and dihydropterin reductase were used to regenerate the tetrahydropterin from dihydropterin (Craine, 1972), allowing $6MPH_4$ oxidation to be measured as a decrease in NADH concentration. Control experiments established that the dihydropterin reductase was not rate limiting and that the hydroxylase lost little or no activity during the experiment.

As can be seen (Figure 1), in the presence of phenylalanine hydroxylase there is an initial rapid burst of oxidation of NADH followed by a very much slower constant rate of oxidation. Extrapolating to zero time showed that the amount of NADH (and thus $6MPH_4$) oxidized in the burst was stoichiometrically related to the amount of phenylalanine hydroxylase present. In the experiment of Figure 1, 0.9 nmol of $6MPH_4$ was oxidized in the burst/nmol of subunit phenylalanine hydroxylase protein (M_r 50 000): determinations with different preparations of enzyme in several separate experiments have shown a range of 0.8–0.9 nmol of $6MPH_4$ oxidized in the burst/nmol of subunit. The same stoichiometric $6MPH_4$ oxidation was found in experiments performed with 0.1 M potassium phosphate (pH 7.8), 0.1 M *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.8), or 0.02 M Tris (pH 8.0) buffers. The enyzme used in these and in all experiments was essentially homogeneous (Shiman et al., 1979).

In further experiments, done as in Figure 1 except with 0.2 μM $6MPH_4$, we found that the addition of 250 μM 6-methyl-5-deazatetrahydropterin to the reaction mixture inhibited the rate of oxidation by 67% and that preincubation of the hydroxylase with 50 μM Dopa inhibited the rate of the oxidation reaction by 97%. The former compound is a competitive inhibitor of the catalytic reaction with respect to $6MPH_4$ (Moad et al. 1979); Dopa is a reversible inactivator of the enzyme.[2]

To establish that neither NADH nor dihydropterin reductase had influenced the results, these two components were omitted, and the $6MPH_4$ concentration was made, in different experiments, 2 or 4 times that of the enzyme; tetrahydropterin oxidation was then monitored directly at 334 nm. When measured this way, a stoichiometry of 1.0 ± 0.1 nmol of $6MPH_4$/nmol of enzyme subunit was found. These experiments confirmed the existence and stoichiometry of the phenylalanine hydroxylase dependent, phenylalanine-independent, oxidation of $6MPH_4$.

When the experiment of Figure 1 was repeated under argon, with buffers and samples which had been degassed in an argon atmosphere to reduce the oxygen concentration, the rate of $6MPH_4$ oxidation was reduced to less than 5% (the limit of precision of the experiment) of that seen at normal oxygen tension (Figure 1, inset). These same degassed samples exhibited a normal stoichiometric $6MPH_4$ oxidation when examined in aerated buffer, indicating that no permanent alteration of the hydroxylase had occurred during the degassing procedure.

Products of the Oxidation Reaction. The results of Figure 1 implied that a quinonoid dihydropterin was formed in the stoichiometric oxidation of $6MPH_4$, since the oxidized pterin product was a substrate for dihydropterin reductase (Kaufman, 1964). This quinonoid dihydropterin could be either a direct product of the reaction or arise by dehydration of a 4a-hydroxydihydropterin. This latter compound is the initial product in catalytic phenylalanine hydroxylation (Lazarus et al., 1981, 1983) and, under the conditions of Figure 1, would be expected to rapidly dehydrate ($t_{1/2}$ = 3.5 s)[2] to the quinonoid species.

[2] J. J. A. Marota and R. Shiman, unpublished observations.

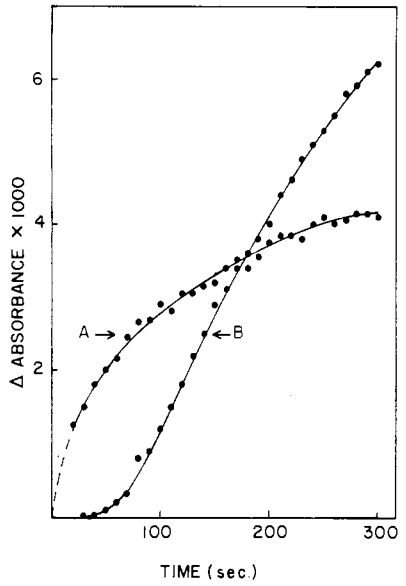


FIGURE 2: Formation of quinonoid-$6MPH_2$ by phenylalanine hydroxylase due to stoichiometric $6MPH_4$ oxidation (A) and catalytic phenylalanine hydroxylation (B). In (A), the reaction mixture contained 1.8 μM phenylalanine hydroxylase and 14 μg/mL catalase in 0.02 M Tris buffer at 18 °C and pH 8.0. The reaction was initiated by the addition of 2.8 μM $6MPH_4$ and was monitored by the change in absorbance at 334 nm. In (B), 0.18 μM phenylalanine hydroxylase, 1.2 mM phenylalanine, and 14 μg/mL catalase were incubated together in 0.02 M Tris, pH 8.0 at 18 °C, for 5 min. At that time, 2.8 μM $6MPH_4$ was added and the reaction monitored at 334 nm. Data are presented as the increase in absorbance at 334 nm at times after the addition of $6MPH_4$.

When the oxidation reaction was performed in 0.02 M Tris at pH 8.0, the dehydration rate could be slowed sufficiently (Lazarus et al., 1981) to distinguish whether the 4a-hydroxydihydropterin or quinonoid dihydropterin was released by the enzyme. Two experiments were performed: In one, the enzyme-dependent oxidation of $6MPH_4$ was monitored at 334 nm. At that wavelength, the quinonoid dihydropterin absorbs strongly ($A_{M,334}$ = 5200 cm^{-1}) while the tetrahydropterin and 4a-hydroxydihydropterin have the same lower absorbance ($A_{M,334}$ = 1300 cm^{-1}). The results, shown in Figure 2, indicate that a quinonoid dihydropterin is directly released by the enzyme. If a 4a-hydroxydihydropterin had been formed, there would have been the appearance of a lag in the reaction progress curve. A lag is seen in a catalytic hydroxylation reaction run as a control under the same buffer conditions (Figure 2). The lag reflects the time required for the nonenzymatic conversion of the 4a-hydroxy to the quinonoid species.

In a second experiment, run under the conditions of Figure 2, the stoichiometric oxidation reaction was monitored fluorometrically by following the dihydropterin reductase linked oxidation of NADH (5 μM NADH and excess dihydropterin reductase were used). (The 4a-hydroxydihydropterin is not a substrate for dihydropterin reductase.[3]) In this, as in the experiment in Figure 2A, there was no appearance of a lag in the reaction progress curve. A control, catalytic phenylalanine hydroxylation reaction, analogous to that in Figure 2B, did show a lag, reflecting the (expected) formation of a 4a-hydroxydihydropterin in the catalytic reaction. The overall conclusion is that within the time constraints of these experiments, the first observable pterin product in the stoichiometric enzyme-dependent oxidation of $6MPH_4$ is a quinonoid dihydropterin.

[3] S. J. Benkovic, Pennsylvania State University, personal communication.

Table I: Formation of Hydrogen Peroxide[a]

order of addition	hydrogen peroxide (RFI)
(1) 0.70 nmol of E_i + 0.35 nmol of $6MPH_4$	<0.2
(2) 0.70 nmol of E_i + 0.35 nmol of $6MPH_4$ + 0.35 nmol of H_2O_2	6.0
(3) 0.70 nmol of E_i + 0.35 nmol of H_2O_2 + 0.35 nmol of $6MPH_4$	6.1
(4) 1.75 nmol of E_i + 0.80 nmol of $6MPH_4$	<0.2

[a] All reactions were run at 25 °C and pH 6.8 in a volume of 2.0 mL which contained, in addition to the indicated components, 0.1 M potassium phosphate. In lines 1 and 4, unactivated phenylalanine hydroxylase (E_i) and $6MPH_4$ were incubated together for 50 s before addition of a solution containing 5.6 μg of horseradish peroxidase and 100 nmol of tyramine. Hydrogen peroxide was detected as an increase in relative fluorescence intensity (RFI) at 405 nm (320-nm excitation) (Zaitsu & Onkura, 1980). In line 2, hydrogen peroxide was added to hydroxylase already preincubated (50 s) with $6MPH_4$, and in line 3, hydrogen peroxide was added to the hydroxylase before the 50-s incubation with $6MPH_4$. In lines 2 and 3, after all additions and incubatins, the peroxidase-tyramine solution was added and the fluorescence determined.

The preceding experiments indicated that $6MPH_4$ was transferring electrons either to phenylalanine hydroxylase, to form a reduced enzyme (or a reduced oxygen–enzyme complex), or to oxygen, to form hydrogen peroxide or superoxide. No evidence, however, for either of these latter two compounds could be found: (1) To test for hydrogen peroxide, $6MPH_4$ and phenylalanine hydroxylase were incubated together, and hydrogen peroxide was measured fluorometrically (Zaitsu & Ohkura, 1980). No detectable hydrogen peroxide was released from the enzyme in the reaction (Table I). Authentic hydrogen peroxide added just before or just after $6MPH_4$ oxidation was easily detected (Table I). (2) To test for the superoxide anion, superoxide dismutase was added (7–33 units/mL) to the reaction mixture and the experiments of Table I repeated. [Superoxide dismutase will convert two superoxide anions to a hydrogen peroxide plus an O_2 (McCord & Fridovich, 1969).] Again, no hydrogen peroxide was detected, indicating that superoxide was also not present. The implications of these experiments is that phenylalanine hydroxylase, with or without a bound oxygen, must be the electron acceptor; that is, the enzyme appears in a functional sense to be reduced by $6MPH_4$. As will be detailed below, this electron transfer to phenylalanine hydroxylase (i.e., reduction of the enyzme) results in an enzyme that is chemically, physically, and catalytically altered.

Stability of the Reduced State. It was important to know whether the electrons transferred to the enzyme from $6MPH_4$ could be used in any direct way to drive phenylalanine hydroxylation. This was shown not to be the case by incubating enzyme first with $6MPH_4$ and then with [^{14}C]phenylalanine (Table IIA). Almost no [^{14}C]tyrosine was formed. Control experiments indicated (1) that the little tyrosine that is formed reflects unoxidized $6MPH_4$ remaining at the time of phenylalanine addition, (2) that throughout the experiment the enzyme retained its catalytic activity, and (3) that nonenzymatic oxidation accounted for at most 3–5% of the $6MPH_4$ consumed.

We have previously shown that phenylalanine hydroxylase must be activated with phenylalanine to be catalytically competent (Shiman & Gray, 1980). In the experiments of lines 1–4 (Table IIB), the enzyme was incubated with excess phenylalanine to give an activated enzyme (E_a), and then

Table II: Oxidation of $6MPH_4$ by Phenylalanine Hydroxylase[a]

line	enzyme form	enzyme amount (nmol)	reaction time (s)	$6MPH_4$ added (nmol)	[^{14}C]tyrosine formed (nmol)
part A					
1	E_i	1.65	75	1.14	0.065 ± 0.005
2	E_i	1.65	150	1.14	0.075 ± 0.005
3	E_a	0.08		1.14	0.98
part B					
1	E_a	1.3	60	0.65	0.34
2	E_a	1.3	30	0.65	0.31
3	E_a	1.3	60	1.30	0.89
4	E_a	1.3	30	1.30	0.84
5	E_a^*	1.3	60	1.30	1.30
6	E_a^*	1.3	60	0	0.01

[a] (A) In lines 1 and 2, unactivated phenylalanine hydroxylase (E_i) was incubated in 0.5 mL of 0.024 M potassium phosphate buffer and 14 μg/mL catalase, at pH 6.8 and 25 °C, with $6MPH_4$ for the indicated times prior to addition of [^{14}C]phenylalanine to 0.5 mM. After 6 additional min the reaction was stopped by a addition of acid (Miller et al., 1975) and [^{14}C]tyrosine determined by the crystallization method of Miller et al. (1975). Tyrosine formed is the average of duplicate determinations. In line 3, the [^{14}C]phenylalanine was added to the enzyme and the enzyme allowed to activate for 2.5 min to form E_a prior to addition of $6MPH_4$. (B) In lines 1-4, phenylalanine hydroxylase was incubated for 2.5 min in 0.4 mL of 0.5 mM phenylalanine, 0.1 M potassium phosphate, and 4 μg/mL catalase, all at pH 6.8 and 25 °C, to form activated enzyme (E_a). In lines 5 and 6, the enzyme was incubated with 1.3 nmol of $6MPH_4$ for 2.25 min prior to addition of the phenylalanine to the buffer; the sample was then incubated an additional 2.5 min to yield reduced, activated enzyme (E_a^*). In all cases except line 6, after activation with phenylalanine, [^{14}C]phenylalanine and the indicated $6MPH_4$ were added in that order. Th reaction was stopped with acid and [^{14}C]tyrosine determined as in (A). "Tyrosine formed" is the average of duplicate determinations.

Table III: Hydrogen Peroxide Inactivation of Reduced Phenylalanine Hydroxylase

(A) Incubation of Enzyme with $6MPH_4$[a] amount present during preincubation

E_i (μM)	catalase (μg)	$6MPH_4$ (μM)	7,8-$6MPH_2$ (μM)	activity remaining (%)
0.64				91
0.64		4.0		17
0.64			4.0	89
0.64	15.3	4.0		90
0.64	15.3			86

(B) Incubation of Reduced Enzyme with Hydrogen Peroxide[b]

E_i (μM)	catalase (μg)	H_2O_2 (μM)	enzyme activity (nmol of Tyr/min) [$6MPH_4$] in preincubation: 0	2.7 μM	control
0.52		50	20.2	0.6	21.5
0.52		20	21.0	0.7	20.3
0.52	16	20	21.9	20.9	21.5

[a] Identical aliquots of phenylalanine hydroxylase (E_i) were incubated at 25 °C in 250 μL of 0.1 M potassium phosphate buffer, pH 6.8, with the indicated concentrations of catalase, $6MPH_4$, and 7,8-dihydro-6-methylpterin (7,8-$6MPH_2$). After 1 and 5 min of incubation, samples were withdrawn for assay of enzymatic activity (nmol of tyrosine formed/min). Enzyme activity was measured by addition of the preincubated samples as the last component to a standard assay mixture (Shiman & Gray, 1980) containing 10 mM phenylalanine. Data are presented as the percent of enzymatic activity remaining after a 5-min incubation. The 7,8-dihydropterin was generated by permitting 4.0 μM $6MPH_4$ to oxidize in the buffer for 60 min before the enzyme was added. [b] Phenylalanine hydroxylase (E_i) was incubated in 225 μL of 0.02 M potassium phosphate buffer, pH 6.8, for 2 min at 25 °C with the concentrations of $6MPH_4$ and catalase indicated. Hydrogen peroxide was then added; after 60 s a sample was withdrawn for immediate assay of activity (nmol of tyrosine formed/min) as in (A). Control preincubations did not contain $6MPH_4$ or hydrogen peroxide; proportionate amounts of these compounds were added to the completed assay mixture.

$6MPH_4$ was added and tyrosine production measured. As can be seen, a significant fraction of the $6MPH_4$ was not available for tyrosine formation. When the enzyme was reduced with an equivalent of $6MPH_4$ before addition of the phenylalanine and then a second addition of $6MPH_4$ made, the reaction now yielded one tyrosine/$6MPH_4$ oxidized (line 5, Table IIB). A control reaction done in the same way, but without the second addition of cofactor, gave, as expected, no tyrosine (Table IIB). These data imply that the rate of "nonproductive" $6MPH_4$ consumption by phenylalanine-activated enzyme (presumptively, enzyme reduction) is of the same magnitude as the catalytic rate and that the reduced state is relatively stable, even in a catalytically turning over enzyme. This latter observation implies that the enzyme need be reduced only once.

Hydrogen Peroxide Inactivation. Incubation of phenylalanine hydroxylase in the absence of catalase with even very low levels of $6MPH_4$ leads to inactivation of the enzyme (Table IIIA). The implication is that hydrogen peroxide, arising from nonenzymatic tetrahydropterin oxidation, will inactivate the enzyme. [Phenylalanine hydroxylase inactivation by hydrogen peroxide formed in this way was reported some time ago (Nielsen, 1969); in that case a 50-fold greater concentration of pterin was used.]

Table IIIB shows that $6MPH_4$-reduced enzyme is much more sensitive to inactivation by hydrogen peroxide than nonreduced enzyme. If treated with excess $6MPH_4$, virtually all enzyme can be inactivated. These results indicate that $6MPH_4$ induces a change in chemical reactivity of the enzyme and that all catalytically active enzyme molecules are subject to this change. The implication is that all catalytically active enzyme has been reduced by $6MPH_4$.

Effects on Enzyme Fluorescence and UV–Visible Absorption Spectra. After reduction with $6MPH_4$, phenylalanine hydroxylase shows an increased fluorescence intensity and a shift in the fluorescence emission maximum (Figure 3). For the spectra in Figure 3, the oxidized $6MPH_4$ was removed from the enzyme by gel-exclusion chromatography; however, identical fluorescence changes are seen if the pterin is not separated from the enzyme.[4] Phenylalanine-activated enzyme also shows an increase in fluorescence intensity when $6MPH_4$ is added, and identical spectra are obtained whether the enzyme is phenylalanine activated and then exposed to $6MPH_4$ or exposed to $6MPH_4$ and then activated with phenylalanine (not shown).[5]

Reduction also induces a change in the absorption spectrum of phenylalanine hydroxylase (Figure 4). A decreased absorbance is evident throughout the spectrum, with the largest relative changes being seen in the broad shoulder that extends from 300 nm into the visible region. [This long shoulder in

[4] Pterin and enzyme were completely separated by this procedure, and UV spectral evidence excluded the possibility of any significant amount of oxidized pterin having remained tightly bound to the enzyme.

[5] Concurrent with phenylalanine activation is an increase of about 15 nm in the enzyme's fluorescence emission maximum. (D. Gray, S. Jones, and R. Shiman, unpublished results). Reduction induces the same quantitative increase in the fluorescence intensity of the spectra of activated as unactivated enzyme.

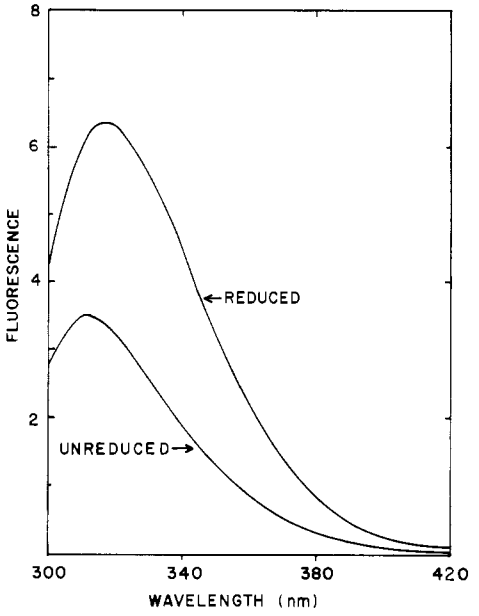


FIGURE 3: Fluorescence emission spectra of reduced and nonreduced phenylalanine hydroxylase. The "reduced" enzyme was obtained by incubating a sample of phenylalanine hydroxylase with 2 equiv of $6MPH_4$ in 0.024 M potassium phosphate buffer containing 14 μg/mL catalase, all at pH 6.8 and 25 °C in 0.6 mL total volume. After 2 min, KCl was added to 0.1 M, and the pterin was removed by rapid chromatography of the sample on a 2.5-mL bed (1 × 3.3 cm) of Bio-Gel P4 equilibrated and eluted with the phosphate–catalase incubation buffer. The protein-containing fractions were pooled, and the spectrum was immediately obtained. The "unreduced" enzyme spectrum was obtained from a sample treated in an identical way as above except that $6MPH_4$ was omitted from the initial incubation. After chromatography the concentration of protein for both the reduced and unreduced sample was 0.40 ± 0.01 mg/mL, as determined by the method of Lowery et al. (1951) with crystalline bovine serum albumin as the standard. For these spectra, the samples were diluted in phosphate–catalase incubation buffer to 0.12 mg/mL; the excitation wavelength was 275 nm, and a band-pass of 2 nm was used for both the emission and excitation monochromators. Spectra were obtained at 25 °C. In all spectra shown, the spectrum of the phosphate–catalase incubation buffer has been subtracted. "Fluorescence" represents relative fluorescent intensity.

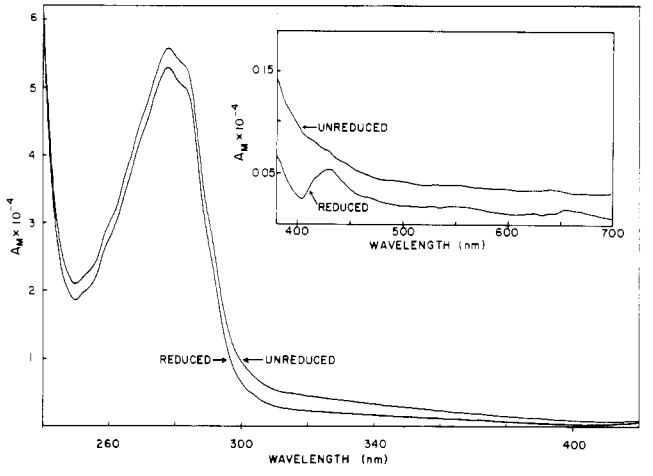


FIGURE 4: UV–visible spectra of reduced and nonreduced phenylalanine hydroxylase. The same samples were used as in Figure 3, but without dilution, i.e., at a concentration of 0.40 mg/mL. The absorption spectra extinction coefficients, A_M, are calculated per subunit and are based on a M_r (subunit) 50 000. The absorbance of the phosphate–catalase incubation buffer has been subtracted from the spectra shown. Spectra were obtained at 25 °C with a 1-nm band-pass.

the spectrum of normal, nonreduced enzyme has been reported (Fisher et al., 1972); it was suggested at that time it might

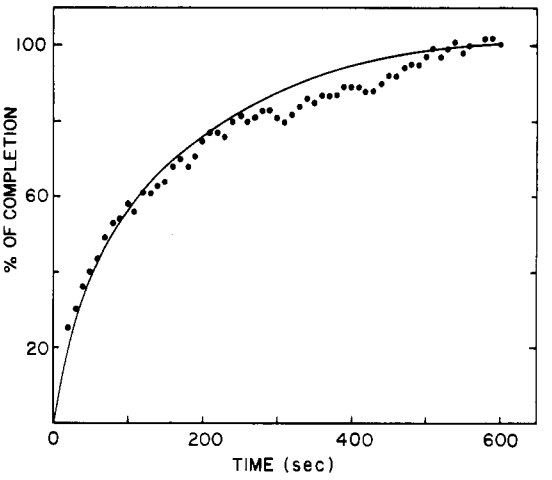


FIGURE 5: Correlation fluorescence change with $6MPH_4$ oxidation. Time course of phenylalanine hydroxylase dependent oxidation of $6MPH_4$ (●) and $6MPH_4$-induced increase in phenylalanine hydroxylase fluorescence intensity (solid line) determined under identical conditions. The reactions were performed at 18 °C in 0.02 M Tris, pH 8.0. The complete reaction mixture contained, in addition to buffer, 14 μg/mL catalase, 1.8 μM phenylalanine hydroxylase, and 2.8 μM $6MPH_4$; reactions were initiated by the addition of $6MPH_4$ as the last component. Enzyme fluorescence intensity was monitored at 330 nm (275-nm excitation). Oxidation of $6MPH_4$ was monitored as the formation of quinonoid-$6MPH_2$ spectrophotometrically at 334 nm; values have been corrected for nonenzymatic $6MPH_4$ oxidation. The increase in fluorescence intensity and $6MPH_4$ oxidation are presented as the percent of completion of the reaction at times after addition of the $6MPH_4$; 600 s was taken as the end point.

be due to the non-heme iron (Fisher et al., 1971; Gottshall et al., 1982) that is present in phenylalanine hydroxylase.] After reducton, a new broad peak of unknown significance is evident at 415 nm. It should be noted that the differences shown in Figure 4 are fairly large and, as a result, must be taken into account when interpreting changes in absorbance resulting from an enzyme–tetrahydropterin reaction.

The same samples were used for the spectra of Figures 3 and 4, and both spectral methods show a $6MPH_4$-induced alteration of the properties of the enzyme, demonstrating that the protein has been physically affected by reduction. The absorbance and fluorescence changes appear to be related, and it is possible that the strong iron absorbance (or the iron's physical state) in the nonreduced protein is responsible for a quenching of protein fluorescence. [Independently and concurrently, Wallick et al. (1984) have discovered that $6MPH_4$ affects the chemical and physical state of the non-heme iron bound to phenylalanine hydroxylase.]

Enzyme Fluorescence, Enzyme Reduction, and Dopa Inhibition. In Figure 5, the rate and extent of fluorescence increase and $6MPH_4$ oxidation are shown to be identical within experimental error. This experiment was performed in Tris buffer at pH 8.0. Related experiments in 0.1 M phosphate buffer at pH 6.8 also showed an exact correlation between $6MPH_4$ oxidation and the increase in the non-phenylalanine-activated enzyme fluorescence.

As indicated above, we had noticed that Dopa inhibited both the enzyme-induced stoichiometric oxidation of $6MPH_4$ and the activity of the enzyme. Figure 6A shows that preincubation of unactivated enzyme with more than 1 equiv of $6MPH_4$ will prevent these effects of Dopa. That is, reduction and Dopa inhibition in this concentration range are mutually exclusive (competitive) processes. Preincubation of the enzyme with relatively lesser concentrations of $6MPH_4$ resulted in proportionately lesser protection from Dopa inhibition. Of importance in the present context, the ability of $6MPH_4$ to

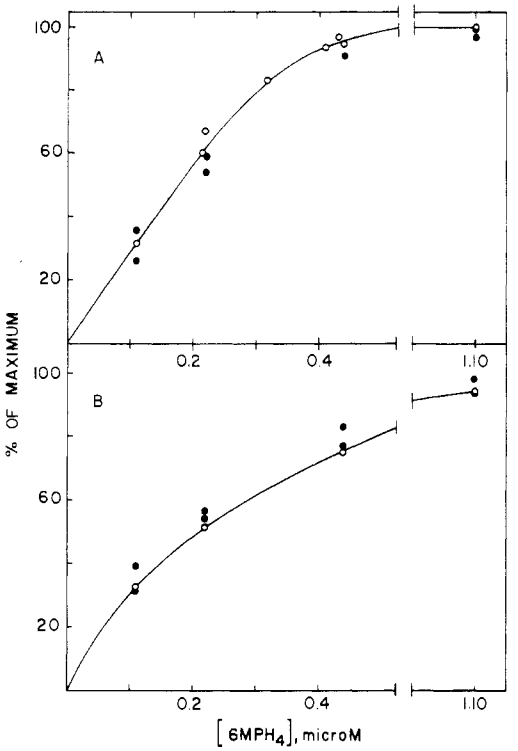


FIGURE 6: Correlation of $6MPH_4$-induced increase in phenylalanine hydroxylase fluorescence (○) with protection from Dopa inhibition (●). All incubations were performed at 25 °C in 0.1 M potassium phosphate buffer, pH 6.8, containing 14 μg/mL catalase. To determine protection from Dopa inhibition, 0.37 μM phenylalanine hydroxylase was incubated for 1.5 min at the indicated $6MPH_4$ concentrations. Dopa was then added to 250 μM and after 30 s an aliquot assayed (performed as in Table II). For the control reactions, a proportional amount of Dopa was added only as the last component to the final complete assay mixture. Enzymatic activity is expressed as a percent of this control value. Enzyme fluorescence was measured in a parallel reaction both before and after addition to $6MPH_4$. The values shown have been corrected for effects of dilution and fluorescence quenching. The results are expressed as a percentage of the increase found with excess $6MPH_4$. (A) Unactivated phenylalanine hydroxylase. Fluorescence excitation 275 nm; emission 330 nm. (B) Phenylalanine-activated phenylalanine hydroxylase. All buffers contained 0.8 mM phenylalanine in addition to the phosphate–catalase buffer; the enzyme was allowed to incubate in this solution for 4 min prior to addition of $6MPH_4$. Fluorescence excitation was 290 nm; emission was 340 nm. All other manipulations were as above.

protect the enzyme from Dopa corresponds to the $6MPH_4$-induced change in enzyme fluorescence for the unactivated (Figure 6A) and also for the phenylalanine activated (Figure 6B) enzyme. That is, the $6MPH_4$-induced fluorescence increase appears to have the same significance for phenylalanine-activated and unactivated enzyme. As a result, the fluorescence change could be used to measure enzyme reduction for both enzyme forms. These data also suggested a possible relationship between enzyme reduction and enzyme activity.

When the change in fluorescence was used as a measure of enzyme reduction, second-order rate constants of reduction, k_r, were determined for the reactions

$$6MPH_4 + E_i \xrightarrow{k_r} E_i^* + 6MPH_2$$

$$6MPH_4 + E_a \xrightarrow{k_r} E_a^* + 6MPH_2$$

where E_i^* and E_a^* represent reduced forms of unactivated and phenylalanine-activated enzyme, respectively. Table IV shows that phenylalanine-activated enzyme is more rapidly reduced than unactivated enzyme and that lysolecithin, which will also

Table IV: Second Order Rate Constants for $6MPH_4$ Reduction of Phenylalanine Hydroxylase[a]

Part A
$k_r \times 10^{-6}$ (min^{-1} M^{-1})

	0.02 M phosphate, pH 6.8		0.02 M Tris, pH 8.0	
enzyme	25 °C	10 °C	25 °C	10 °C
E_i	11	1.2	0.7	0.1
E_a	15	8.0	3.1	1.8
E_a(lyso)				2.8

Part B
$k_r \times 10^{-6}$ (min^{-1} M^{-1}) for phenylalanine-activated enzyme at 25 °C

buffer	pH 7.5	pH 8.0	pH 8.5
0.02 M Tris	7.2	3.6	2.0
0.02 M Tris + 0.1 M potassium phosphate		3.3	1.5
0.04 M Tris		1.8	1.0

[a] Reduction was monitored as an increase in phenylalanine hydroxylase fluorescence intensity at 340 nm (290-nm excitation) for the unactivated (E_i), phenylalanine-activated (E_a), and lysolecithin-activated [E_a(lyso)] enzyme. (Part A) The reaction mixtures contain 0.24 μM phenylalanine hydroxylase, 0.65–1.25 μM $6MPH_4$, and 14 μg/mL catalase in 1.7 mL of the indicated buffer and pH; reaction temperature was maintained at either 25 or 10 °C. For determinations with activated enzyme, the reaction mixture contained, in addition, 1.2 mM phenylalanine or 1.2 mM lysolecithin; for reactions at 25 °C, the enzyme was added to the reaction mixture 4 min prior to addition of $6MPH_4$; for reactions at 10 °C, the enzyme was activated at 25 °C, with 1 mM phenylalanine or lysolecithin in a buffer containing 0.02 M potassium phosphate, pH 6.8, and 14 μg/mL catalase. An aliquot of these activated samples was added to a reaction mixture at 10 °C after which $6MPH_4$ was added to initiate the reaction. The initial portion of the reaction curve could be analyzed as a pseudo-first-order reaction. (Part B) Second-order rate constants for the reduction of phenylalanine-activated phenylalanine hydroxylases were determined as described above for part A. The complete reaction mixture contained at 25 °C, in 1.7 mL, buffer and pH as indicated, 14 μg/mL catalase, 0.22 μM phenylalanine hydroxylase, 1.2 mM phenylalanine, and 0.82 μM $6MPH_4$. Enzyme was phenylalanine activated by preincubation for 2.5 min in the reaction mixture before reduction was initiated by addition of $6MPH_4$ as the last component.

activate the enzyme (Shiman & Gray, 1980), has an effect similar to that of phenylalanine on the rate of reduction. The reaction of enzyme and $6MPH_4$ appear, within experimental error, second order up to 2 μM $6MPH_4$; higher concentrations were not tested. [For comparative purposes, based on the enzyme turnover rate (Shiman et al., 1982) and a K_M for $6MPH_4$ of 45 μM, a second-order rate constant, k_c, for the reaction of $6MPH_4$ and enzyme during catalysis can be calculated. The value obtained for k_c is about 28×10^6 min^{-1} M^{-1} in 0.1 M potassium phosphate buffer, pH 6.8 and 25 °C.]

In Tris buffers, the reduction process is greatly slowed relative to what one would find in a phosphate buffer; the free base form of Tris appears to be the major inhibiting species. Sufficiently high Tris concentrations will almost completely block reduction. Although not shown, pH has little effect on k_r when phosphate buffers are used, and phosphate itself does not increase k_r (Table IV). In a practical way, these data show that k_r can be varied at will over a wide range by simple manipulations of pH, temperature, and buffer.

Effect of Tris on the Catalytic Reaction. We have found that Tris also inhibits the catalytic activity of phenylalanine hydroxylase (Figure 7). In these experiments, the enzyme was both reduced with $6MPH_4$ and activated with phenyl-

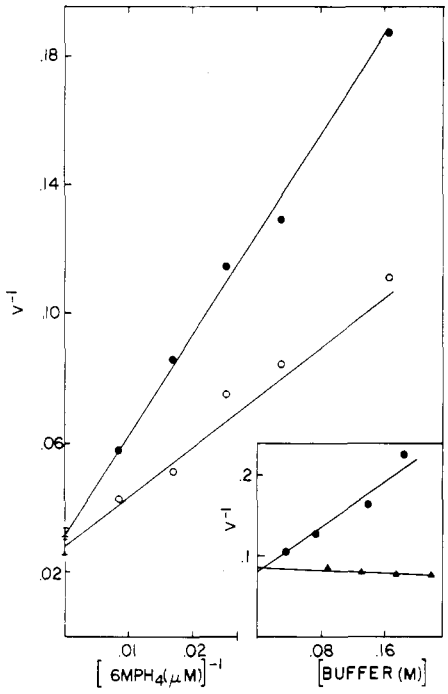


FIGURE 7: Inhibition of catalysis by Tris. The data are presented as a plot of V^{-1} [velocity (V) is as nmol of Tyr formed/min] vs. $[6MPH_4\ (\mu M)]^{-1}$ with no (○) or 0.2 M Tris (●). The data have been fit to a hyperbola according to Wilkinson (1961). The reaction mixture contained $6MPH_4$ and Tris as indicated, 2 mM phenylalanine, 6 mM dithiothreitol, 60 μg of catalase, and 0.1 M potassium phosphate, all at pH 7.8 and 25 °C. The reaction was initiated by addition of reduced, activated phenylalanine hydroxylase (see below). Tyrosine formation was measured spectrophotometrically at 275 nm (Shiman & Gray, 1980). (Inset) A plot of V^{-1} vs. buffer concentration. Tris (●) and potassium phosphate (▲) buffers are used at pH 7.8 and 25 °C, as indicated, and other components were as above with a $6MPH_4$ concentration of 20 μM in all reactions. KCl was added to maintain ionic strength at 0.68 M for phosphate buffer and 0.37 M for Tris buffer. Reduced activated phenylalanine hydroxylase (see below) was added last to initiate the reaction. The reaction rate was measured by $6MPH_4$ oxidation at 334 nm (A_M = 3850 cm^{-1}) (Kaufman, 1971). To reduce and phenylalanine activate phenylalanine hydroxylase, the sample, in 0.02 M potassium phosphate buffer containing 14 μg/mL catalase at pH 6.8 and 25 °C, was first made 14 μM in $6MPH_4$, and then 1.5 min later, phenylalanine was added to 12.5 mM; after 0.5 min the sample was added to the reaction mixture. For assays in Tris, excess deglycerinated dihydropterin reductase was added: the partially purified preparation catalyzes the conversion of 4a-hydroxydihydropterin to quinonoid dihydropterin.[3]

alanine prior to its addition to the assay mixture. The inhibition appears to be competitive with respect to $6MPH_4$ (Figure 7) with an apparent K_I (slope) of about 0.08 M Tris under the given conditions. Neither phosphate (inset, Figure 7) nor ionic strength has an appreciable effect on the catalytic rate at this pH and temperature. A pH dependence of inhibition, similar to that observed with the reduction reaction, is also observed with the catalytic reaction; that is, the higher the pH the more effective Tris is as an inhibitor: at pH 6.8, even 0.2 M Tris has almost no effect on the reaction rate (not shown). It is probable from these results that again the free base form of Tris is the inhibitor. It should be noted that Tris is often used as a buffer in assays of this enzyme (Bailey & Ayling, 1980; Huang et al., 1973; Lazarus et al., 1981), and its effect as an inhibitor has never been recognized and taken into account. Glycerol, which to some degree structurally resembles Tris, will also inhibit both enzyme reduction and catalysis. Again its effect appears to be competitive with $6MPH_4$ in both processes.[2]

Effect of Reduction on Catalysis. The most important question arising from the preceding observations concerns the

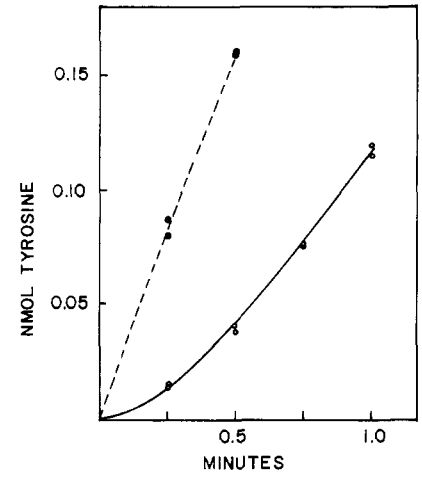


FIGURE 8: Time course of tyrosine formation by prereduced (●) and initially nonreduced (○) phenylalanine hydroxylase. The solid line (—) is a calculated rate of formation of tyrosine based on the assumption that phenylalanine hydroxylase must be reduced to be catalytically active (see Materials and Methods). The dashed line (- - -) is a line connecting the average values of the data points for the $6MPH_4$-preincubated samples. The reaction mixture contained, in 0.5 mL volume, 0.04 M Tris, pH 8.5, 14 μg/mL catalase, 0.1 mM phenylalanine 24 × 10^5 cpm of [^{14}C]phenylalanine, 1 μM $6MPH_4$, and 0.08 nmol of phenylalanine hydroxylase. Identical aliquots of enzyme were activated by incubation with 0.25 mM phenylalanine for 2 min in 0.2 mL of catalase-containing buffer; samples were then incubated an additional 2 min either with addition of 0.5 nmol of $6MPH_4$ to form prereduced activated enzyme or without addition of $6MPH_4$ leaving nonreduced activated enzyme. [^{14}C]Tyrosine formation was initiated by addition of 0.3 mL of buffer and a solution containing [^{14}C]phenylalanine and 0.5 nmol of $6MPH_4$. Reactions were run at 25 °C for the indicated times and then stopped by addition of acid (Miller et al., 1975). [^{14}C]Tyrosine was determined by the crystallization method of Miller et al. (1975).

functional significance of enzyme reduction. Results in Table IV and Figure 5 suggested that reduction could be a prerequisite for catalytic activity, but the evidence was indirect. To test this possibility more directly, reaction conditions were chosen (based on the data in Table IV and Figure 7) such that the rate of reduction was much slower than the catalytic rate. As shown in Figure 8, under these conditions, the catalytic activity of initially nonreduced enzyme shows a pronounced lag in the rate of tyrosine formation. By contrast, prereduced enzyme shows no lag in the rate of tyrosine formation.

The solid line in Figure 8 is calculated rate of tyrosine formation based on the assumption that phenylalanine hydroxylase must first be reduced to be catalytically active. Specifically, the solid line is drawn in accordance with the following kinetic model:

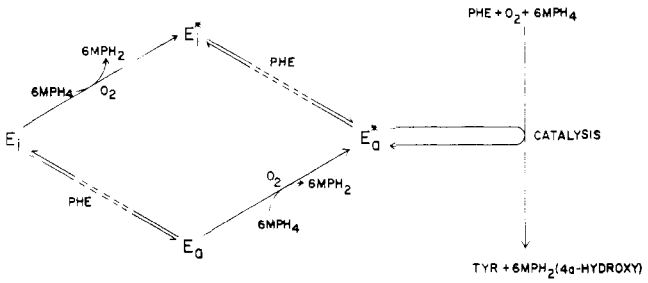
$$E_a + 6MPH_4 \xrightarrow{k_r} 6MPH_2 + E_a^*$$

$$E_a^* + Phe + O_2 + 6MPH_4 \xrightarrow{k_c} 6MPH_2 + Tyr + E_a^*$$

For the calculations, the value of k_c, the rate constant for catalysis, was determined from the rate of the reaction of the prereduced enzyme (Figure 8); k_r, the second-order rate constant of reduction, was determined from the rate of increase in fluorescence intensity of initially unreduced phenylalanine hydroxylase under conditions identical with those of Figure 8. As can be seen (Figure 8), the fit of the data to the model is extremely good. The implication is that phenylalanine hydroxylase must be reduced by $6MPH_4$ to form a catalytically active enzyme species.

In the experiment of Figure 8, when all the 0.5 nmol of $6MPH_4$ added to the reactions was consumed, the reaction

Scheme I



with the prereduced enzyme yielded 0.47 nmol of tyrosine and that with the initially nonreduced enzyme 0.39 nmol of tyrosine. The difference between the two is almost exactly equal to the 0.08 nmol of phenylalanine hydroxylase (subunits) used in the reaction and reflects the cofactor required to reduce the initially nonreduced enzyme. This result is completely consistent with data presented earlier.

Discussion

This paper presents evidence that rat liver phenylalanine hydroxylase will oxidize $6MPH_4$ in a stoichiometric reaction that results in an enzyme that is chemically and physically altered. Furthermore, and of greatest importance, this alteration is required for the formation of a catalytically active enzyme. These results are summarized in Scheme I. E_a^*, the phenylalanine-activated $6MPH_4$-reduced enzyme, is the only catalytically active species. In the absence of additional tetrahydropterin, E_a^* is not itself capable of driving phenylalanine hydroxylation. That is, this reduction appears completely different from reductions observed with certain other enzymes, such as dopamine β hydroxylase (Friedman & Kaufman, 1965), in which electrons transferred to the enyzme from a cofactor can be used directly in a second step for a hydroxylation reaction. In addition, as reflected in the rate constants of reduction, activated enzyme (E_a) appears to be more rapidly reduced than unactivated enyzme (E_i), indicating that activation by either phenylalanine or lysolecithin affects the reduction site as well as the catalytic site. It is possible that the reduction site and the site at which tetrahydropterin binds during catalysis are the same, and in support of this possibility, Tris and glycerol are inhibitors of reduction and also independently of catalysis. Although direct experiments are necessary to better define their relationship, it is clear from these data that, at the least, the two sites have quite similar properties.

The stoichiometry of enzyme-induced phenylalanine-independent $6MPH_4$ oxidation is an important characteristic and has been determined in several different ways: by linking the reaction to NADH oxidation (Figure 1), by directly measuring the absorbance increase due to quinonoid dihydropterin formation, by fluorescence titration (Figure 6A), and by measuring the effect of reduction on Dopa inhibition (Figure 6A). All four methods agree. The mean value from all methods (about 20 independent determinations) is that 0.95 ± 0.1 $6MPH_4$ is oxidized per enzyme subunit.

In what appears to be a contradiction but in fact is not, Wallick et al. (1984) have shown that in the presence of *o*-phenanthroline, the addition of 0.5 equiv of $6MPH_4$/enzyme subunit allows the release of all enzyme-bound iron as an *o*-phenanthroline–Fe^{2+} complex. (We have confirmed this result.) However, to obtain their result, it is critical that the *o*-phenanthroline be added to the enzyme prior to addition of $6MPH_4$. If the order of addition is reversed, with $6MPH_4$ being allowed to reduce the enzyme before addition of *o*-phenanthroline, little iron (<20% of the expected amount) is released from the enzyme as an *o*-phenanthroline complex.[2,6] This latter result shows that in the presence of oxygen and the absence of an amphiphathic chelator such as *o*-phenanthroline, which may itself interact with the enzyme, the tetrahydropterin reduction of the enzyme does more than simply change the oxidation state of the bound iron. Thus, at present there is no obvious disagreement between our results and those of Wallick et al. (1984), and all available evidence indicates that in the presence of oxygen and the absence of inhibitors of catalytic activity, one tetrahydropterin is required to reduce each phenylalanine hydroxylase subunit. However, one must recognize that in the strictest sense, this stoichiometry only refers to unactivated enzyme. A reliable reduction stoichiometry for phenylalanine-activated enzyme is difficult to determine and is not yet known.

The fate and role of the oxygen required in the reduction step in the above scheme is not clear. Our inability to detect a reduced oxygen species in solution after reduction of the enzyme with $6MPH_4$ and unpublished[2] indirect experiments support the possibility that some form of oxygen is bound to the enzyme during reduction. The "bound" oxygen could be usable during catalysis and in some sense represent an activated oxygen species or could have another unknown function. The answer to this is of great importance for an understanding of the mechanism of the reduction step and possibly of the catalytic cycle as well.

Additional questions of great interest concern the nature of the functional groups on the enzyme that accept the electrons from the tetrahydropterin [see Wallick et al. (1984)] and the reversibility of the reduction process. Although we have not yet answered the first question, we have been able to "recover" electrons from reduced enyzme with a concurrent reversal of the enyzme alterations.[2] That is, the enzyme is not altered in a permanent, irreversible manner by reduction. We have also found the same reduction of the enzyme with tetrahydrobiopterin, the natural cofactor for this enzyme (Kaufman, 1971), indicating that the phenomenon we are observing is not a peculiarity related to the use of the model cofactor $6MPH_4$.[2]

An electron transfer from the tetrahydropterin cofactor to phenylalanine hydroxylase has been sought but not detected by earlier workers (Kaufman, 1971; Lazarus et al., 1981). The effect was hidden in those cases for different reasons: in one case (Kaufman, 1971), the specific activity of the enzyme employed was very low (less than 10% of that used here); and in the other (Lazarus, 1981), glycerin and/or Tris was present in the experiments. As noted above, these two compounds can each competitively inhibit the rate of enyzme reduction. Once they are removed and the enzyme of high specific activity is employed, the phenomenon is easily seen, as attested to both by the work presented here and the work of Wallick et al. (1984).

That two distinct steps, activation (Shiman, 1980; Shiman et al., 1980) and reduction, are required for the formation of a catalytically active enzyme complicates the study of phenylalanine hydroxylase. At this point, however, effectors of each of the processes or enzyme species have been identified. Specifically, activation can be uniquely inhibited by tetrahydrobiopterin (Shiman et al., 1980), reduction is inhibited by catechols (Dopa is presented in Figure 6), and the reduced enzyme can be irreversibly inactivated by low concentrations

[6] Identical observations have been made in the laboratory of S. J. Benkovic (personal communication).

of hydrogen peroxide (Table III). The mechanisms of action of these compounds in producing these effects are of obvious interest and are currently under investigation, but of most immediate importance, these compounds can now be used as tools for separation of individual events occurring with this enzyme.

It should also be mentioned that both catechol inhibition (Ross et al., 1964; Burkard et al., 1964) and hydrogen peroxide inactivation (Nielsen, 1969) of this enzyme have been known for some time. However, it has not been realized until now that their effects are related to whether or not the enzyme has been exposed to a tetrahydropterin.

Finally, it is possible that the reduction process (or reduced state) is required for catalytic activity of all the unconjugated tetrahydropterin-dependent hydroxylases, that is, for tyrosine and tryptophan hydroxylase as well as phenylalanine hydroxylase. Since neither tyrosine hydroxylase nor tryptophan hydroxylase is as well characterized as phenylalanine hydroxylase and both are relatively difficult to purify, it would at present be difficult to duplicate these experiments with those two enzymes. Nonetheless, the question is important since one would hope (and expect) the three enzymes to have almost identical mechanistic requirements.

Registry No. $6MPH_4$, 942-41-6; phenylalanine hydroxylase, 9029-73-6; hydrogen peroxide, 7722-84-1; Tris, 77-86-1.

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