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Characterization of Phenylalanine Hydroxylase†

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ABSTRACT: Iron can be bound to phenylalanine hydroxylase (PAH) in two environments. The assignment of the electron paramagnetic resonance spectrum of PAH to two, overlapping high-spin ferric signals is confirmed by computer simulation. Both environments are shown to be populated in the crude enzyme. Reconstitution of the apoenzyme demonstrated that the two iron environments are not interconvertible. Oxygen consumption during PAH reduction by tetrahydropterin in the absence of phenylalanine but not in its presence explains the different reduction stoichiometries (tetrahydropterin:enzyme) that have been observed.

Phenylalanine hydroxylase (EC 1.14.16.1) catalyzes the hydroxylation of phenylalanine to tyrosine. Although iron is required for the hydroxylation of phenylalanine (Fisher et al., 1972; Gottschall et al., 1982), the role played by iron in the reaction has only begun to be elucidated. We previously demonstrated (Wallick et al., 1984) that iron is reduced during an obligatory reduction by the pterin cofactor in order to activate the enzyme (Marota & Shiman, 1984). Our work on the EPR[1] of PAH revealed that whereas there is one iron per subunit there are at least two environments for iron in the enzyme; the assignments of the EPR signals to two species with different ligand environments were made as described in Wallick et al. (1984). The magnitude of the signals at g_y and g_x of 6.7 and 5.4 correlates with activity; the magnitude of the broad signal centered around the $g_{eff} = 4.3$ region is inversely

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[1] Abbreviations: PAH, phenylalanine hydroxylase; SDS, sodium dodecyl sulfate; $6MPH_4$, 6-methyltetrahydropterin; $7,8PH_2$, 7,8-dihydropterin; EPR, electron paramagnetic resonance; Phe, phenylalanine; Tyr, tyrosine; 5-thiapterin, 6-phenyltetrahydro-5-thiapterin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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proportional to activity. The interactions of various ligands with the iron center of the enzyme can also be followed by EPR (Wallick et al., 1984).

Here we address the nature of the different iron environments in PAH. Computer simulation is used to examine our previous assignment (Wallick et al., 1984) of the EPR spectrum to two, overlapping high-spin ferric signals. To determine if the iron centers can be interconverted in purified PAH, the iron environments are probed by selective iron removal and replacement. The distribution of iron between the two centers in the unpurified enzyme is determined by measuring the specific activity of PAH in crude homogenates. Perturbation of the iron environments observable by EPR by substrates, cofactors, and inhibitors that function in enzyme activation, prereduction, and inhibition furnishes a direct source of information on the involvement of iron in these aspects of the reaction. The EPR results are related to additional insights into reduction and phenylalanine activation provided by a study of oxygen consumption during reduction.

Experimental Procedures

Buffers. Unless otherwise indicated, the buffer used for elution of columns and for EPR samples contained 0.3 M KCl, 30 mM Tris (pH 7.2, 4 °C), and 50 μM EDTA (elution buffer).

Materials. 6-Phenyltetrahydro-5-thiapterin was prepared by Dr. Robert Henry.

Preparation of Phenylalanine Hydroxylase. PAH was purified from the livers of retired male breeder rats according to Shiman's method through step D (Shiman et al., 1979). The procedure was modified as described by Gottschall et al. (1982) and Shiman et al. (1982). Glycerol was omitted from the DE-52 equilibration and elution buffers as described by Wallick et al. (1984). The purity of the enzyme was shown by SDS gel electrophoresis in 8.75% polyacrylamide (Laemmli, 1970) to be >90%. Protein assays performed with the Peterson modification of the Lowry protein assay (Peterson, 1977) were corrected according to Shiman (1980); an extinction coefficient at 278 nm of 6.8×10^4 M^{-1} was calculated and used in the calculation of protein concentrations. Iron content was determined by atomic absorption; the iron content of the enzyme in these experiments is 1 Fe/50 000-dalton subunit with a variability of 20–30%.

Enzyme Activity Assays. PAH was assayed by observing the change in absorbance at 275 nm due to tyrosine formation (Shiman et al., 1979).

Preparation of Apoenzyme. Apoenzyme was prepared according to the procedure of Gottschall et al. (1982) with the modifications that follow: (1) iron removal was performed with 2–5 mM *o*-phenanthroline, 10 mM DTT, and protein concentrations from 20 to 65 μM; (2) the enzyme was eluted with elution buffer from Sephadex G-25 columns, either 25-mL bed volume columns or 2–4-mL centrifuge desalting columns. Apoenzyme was prepared in high enough concentration that it could be used directly for subsequent steps because concentration of apoenzyme produces an enzyme that cannot be reconstituted to its initial activity.

Preparation of Partial Apoenzyme. The preparation of enzyme from which only the iron that correlates with activity has been removed is essentially the same as the preparation of complete apoenzyme, but the levels of *o*-phenanthroline were lowered to 4–20 equiv/mol of protein.

Reconstitution of Apoenzyme and Partial Apoenzyme. The partial apoenzyme dissolved in elution buffer was reconstituted by adding excess ferrous ammonium sulfate, in a minimal amount of 0.005 N HCl, and incubating at 0 °C for 10 min. It was purified on a Chelex column equilibrated with elution buffer and concentrated on an Amicon Minicon Macrosolute concentrator type A25. The complete apoenzyme was reconstituted with either excess ferrous ammonium sulfate or 2 equiv relative to the protein concentration and purified as described for the partial apoenzyme.

Samples for EPR. PAH was dissolved in elution buffer to prepare EPR samples. Routinely, the sample volume was 0.4 mL. Additions to the EPR samples were made without altering the pH of the solutions. The samples in EPR tubes equipped with serum stoppers were degassed before observation; the only anaerobic addition was the addition of dithionite. Catalase and transferrin were used as signal-intensity standards as described by Wallick ett al. (1984).

EPR Measurements. Paramagnetic resonance spectra were measured with a Varian E109 spectrometer equipped with a low-power microwave bridge (E-102-04), reference arm, and an Oxford Instruments, pumped, liquid helium ESR-10 cryostat and temperature control unit described in Wallick et al. (1984). Samples were measured in quartz EPR tubes with serum stoppers. Reported temperatures for EPR were measured by a thermocouple placed below the sample tube and are thus slightly lower than the actual sample temperature.

Calculation of Specific Activity of PAH in Crude Homogenates. Crude homogenates were prepared as described by Shiman et al. (1979) in homogenization buffer, pH 7.2, containing 0.08 M phenylalanine, 0.2 M KCl, and 0.03 M Tris. Activity was determined as described; activity after iron activation was determined after incubation of the crude homogenate at 4 °C for 20 min in the presence of 2 mM DTT and 0.3 mM ferrous ammonium sulfate according to Shiman and Jefferson (1982). Hydroxylase concentration in the crude homogenates was measured by rocket immunoelectrophoresis (Axelson et al., 1983). Seventeen-milliliter gels of 1% agarose in Monthony buffer (Bio-Rad Laboratories, 1981) containing 110 μL of crude anti-PAH antisera provided by Dr. Ross Shiman were run on 10 cm × 12 cm plates. Purified PAH standards, running to the same height as the PAH in crude homogenates, were used to calculate PAH concentrations in the crudes. The specific activities of multiple samples were averaged since PAH produces diffuse rockets.[2]

Oxygen Consumption during PAH Reduction in the Absence of Phe. Oxygen consumption was monitored on a YSI biological oxygen monitor (Yellow Springs 5301) equipped with a constant-temperature circulator, chart recorder, and a Micro Conversion kit (Yellow Springs 5304). Oxygen uptake was measured when aliquots of $6MPH_4$ dissolved in 0.005 N HCl were added to a solution of PAH, 10 μM in 3.0 mL of 0.1 M potassium phosphate, pH 7.0, incubated at 25 °C (0.25 mM O_2 in solution). For reduction experiments in the presence of catalase, 11 μg/mL catalase was added during the incubation at 25 °C. Hydroxylase activity was measured before and after reduction.

Oxygen Consumption during PAH Reduction in the Presence of Phe. Oxygen consumption during reduction of PAH activated by Phe is difficult to measure because the oxygen consumption owing to product formation has to be factored out. Consequently, these experiments were done at low pterin levels. A 1:1 O_2 consumption to tyrosine formation ratio is indicative of reduction without oxygen uptake as long as it can be shown that a small fraction of reduced hydroxylase is not turning over the entire pterin pool. Computer simulations were done with SIMUL (Barship et al., 1983). Calculations by SIMUL

[2] Ross Shiman, personal communication.

Table I: Specific Activity of Phenylalanine Hydroxylase in Crude Homogenates

PAH homogenate	activity (units/mL)	PAH (mg/mL)	sp act.	sp act. ratio[a]
1	0.94	0.170	5.5	1.1
2	0.68	0.208	3.3	1.2
3	1.0	0.28	3.6	1.1
4	1.1	0.225	4.9	1.0
5	0.88	0.140	6.3	1.1
6	0.68	0.225	3.0	0.87

[a] Specific activity of iron activated/specific activity in the absence of iron addition.

were done on a Vax 11/780 computer under the VMS operating system. The kinetic constants are from Marota and Shiman (1984). Oxygen consumption was monitored when pterin equivalents were added to 10 μM PAH, Phe activated for 3 min at 25 °C in 1.5 mL of 0.1 M potassium phosphate, pH 7.0, containing 1 mM Phe. Tyrosine production was then determined in these samples by the fluorescence of the nitrosonaphthol derivative as described in Wallick et al. (1984). The enzyme activity was measured before and after reduction.

Activity Recovery after Enzyme Reduction. Activity recovery after reduction was monitored by activity assays. Reduction was determined by monitoring the change in absorbance at 334 nm, with the extinction coefficient of 2250 M^{-1} cm^{-1} determined by Marota (1983), when pterin was added to a 10 μM PAH solution in 0.1 M potassium phosphate, pH 6.8. Activity assays were performed over the course of an hour after pterin oxidation was complete. Reduction in the presence of catalase was carried out in the presence of 32 μg/mL catalase.

Results

Specific Activity of PAH in Crude Homogenates. The specific activity of PAH was determined in the crude homogenates of individual rat livers. The specific activities for six different livers are shown in Table I. The activities and protein concentrations given in Table I are representative of more than a dozen trials, with the error in the determination of the hydroxylase concentration being between 20 and 30%. Even if the specific activities are increased by 30%, it is clear that the specific activity of PAH in crude homogenates is still less than the maximal value of 12–14 units/mg (Shiman & Jefferson, 1982). Moreover, iron activation does not increase the activity by more than 10–20%. The relatively low specific activity of PAH in crude homogenates indicates that the iron is already distributed between the active and inactive sites according to our correlation between iron environment and enzyme specific activity (Wallick et al., 1984). If the low activity were due to the presence of apoenzyme, the activity would have been significantly increased by iron activation (Shiman & Jefferson, 1982).

Iron Removal and Reconstitution. Iron was removed from the environment that gives rise to the EPR signal that correlates with activity by using low levels of *o*-phenanthroline. For example, enzyme samples of 0.67 and 0.27 mM were incubated with 3.0 and 5.6 mM *o*-phenanthroline, respectively, to provide the apoenzyme samples for which data are given in Table II. The specific activity of the partial apoenzyme is less than 20% of the specific activity of the holoenzyme. The amount of iron visible at g_{eff} = 6.7 and 5.4 in the apoenzyme is approximately 15% that in the holoenzyme (Table II). The specific activities of the holo- and apoenzymes correlate inversely with the integrated iron intensities of the broad component centered around g_{eff} = 4.3 (Wallick et al., (1984). The

Table II: Removal of Iron from Phenylalanine Hydroxylase and Enzyme Reconstitution[a]

	sp act.	iron content[b]	relative EPR ratios[c]
holoenzyme	5.5, 7.0	1.1, 0.7	1, 1
apoenzyme	0.6, 1.5	0.6, 0.3	0.15, 0.1
reconstituted enzyme	5.0, 7.9	1.1	0.76

[a] Data are given for two separate samples. In the second preparation, the reconstituted enzyme was not purified to remove excess g_{eff} = 4.3 iron so iron content and EPR ratios were not determined. [b] The estimated error in iron determination by the atomic absorption is ±0.2. Units are moles of iron per mole of protein subunits. [c] The ratio of $g_{eff} \simeq 6.7/g_{eff} \simeq 4.3$ relative to the holoenzyme. This ratio was determined by measuring the vertical distance between the maximum at 6.7 and the minimum at 6.3 relative to a similar maximum to minimum distance in the g_{eff} = 4.3 region and also by base-line subtraction and double integration (Wallick et al., 1984).

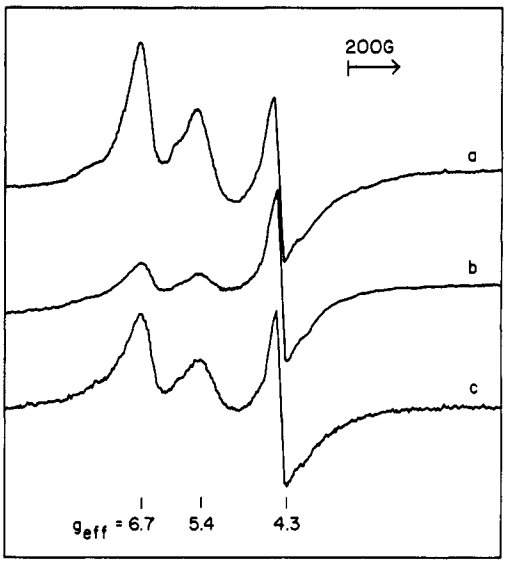

FIGURE 1: Low-field region of the X-band EPR spectra of PAH at 5 K after partial iron removal and replacement. (a) EPR spectrum of native PAH (160 μM, sp act. = 5.5, 1.1 iron/subunit). Instrument settings as follows: modulation amplitude 20 G; receiver gain 1 × 10^4; microwave power 0.1 mW; time constant 0.25 s; scan time 8 min; scan range 0.4–2.4 KG. Values of prominent spectral features are given. (b) EPR spectrum of PAH (170 μM, sp act. = 0.6, 0.6 iron/subunit) after partial iron removal. Instrument settings are described in (a). (c) EPR spectrum of the sample described in (b) after reconstitution. The 88 μM reconstituted sample has sp act. = 5.0 and 1.1 iron/subunit. The instrument settings described for (a) were used except that the gain is 2 × 10^4.

correlation is not as satisfactory for the reconstituted enzyme (90% activity recovery vs. 76% recovery of g_{eff} = 6.7 and 5.4 intensity). A factor contributing to this discrepancy is the lower concentration of protein and consequent greater uncertainty in integrating the EPR spectrum of the reconstituted enzyme. The EPR spectra for the three enzyme forms are displayed in Figure 1. They show that, at low levels of *o*-phenanthroline, iron is preferentially removed from the site that gives rise to g_{eff} = 6.7 and 5.4 and this same site is repopulated in the reconstitution step.

All the iron can be removed from the enzyme (20–65 μM) with higher levels (2–7 mM) of *o*-phenanthroline as shown in the EPR spectrum (Figure 2) of the apoenzyme prepared under these conditions. Reconstitution of this enzyme leads to repopulation of both iron sites as shown in Figure 2. On average, 90% of the initial specific activity is restored. We cannot comment on the relative intensities of the two types of EPR signals in this particular case because the sample used for Figure 2 was not subjected to the usual iron activation step

Table III: Parameters Used To Simulate Phenylalanine Hydroxylase EPR Spectrum[a]

	ratio of ZFS, λ	D (cm^{-1})	effective g values			fractional contribution (%)
			g_x	g_y	g_z	
site I (lower doublet)	0.032	1.0	5.4	6.7	2.0	37
site II (middle doublet)[b]	(λ_0 = 0.20, $\Delta\lambda$ = 0.05)	0.3				55
site III (middle doublet)	0.30	0.28	4.1	3.9	4.4	5
site IV (middle doublet)	0.33	0.37	4.2	4.2	4.2	3

[a] ZFS = zero-field splitting. The microwave frequency used in the simulation was 9.3 GHz. Line shapes were frequency-swept Gaussian functions for sites I, III, and IV (A. S. Yang and B. J. Gaffney, submitted for publication). [b] This broad signal was simulated with a Gaussian distribution of λ values ($\exp[-(\lambda - \lambda_0)^2/[2(\Delta\lambda)^2]]$). This distribution was centered around λ_0 = 0.2 and had $\Delta\lambda$ = 0.05. A distribution of λ values means there is also a distribution of effective g values so unique g values cannot be given for site II.

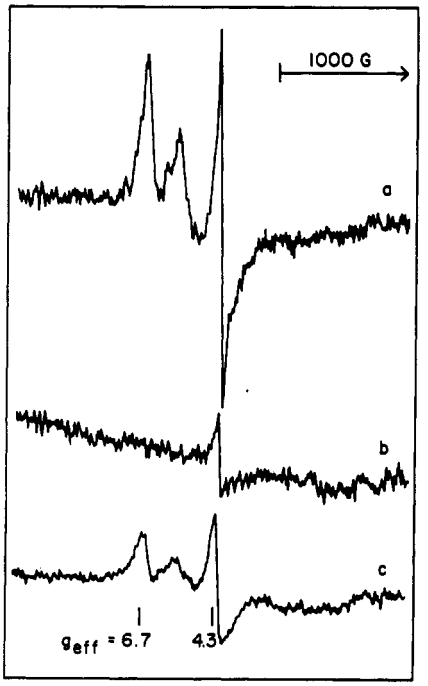

FIGURE 2: EPR spectra at 5 K of apoenzyme and the reconstituted enzyme. (a) EPR spectrum of PAH (58 μM, sp act. = 3.4) at 5 K. The instrument settings are those given in Figure 1a except that receiver gain is 4 × 10^4. (b) EPR spectrum of apoenzyme (54 μM, sp act. = 0.45). Instrument settings are the same as in (a). (c) EPR spectrum of the sample shown in (b) after reconstitution (30 μM PAH). The instrument settings are those given in Figure 1 except for gain = 2 × 10^4.

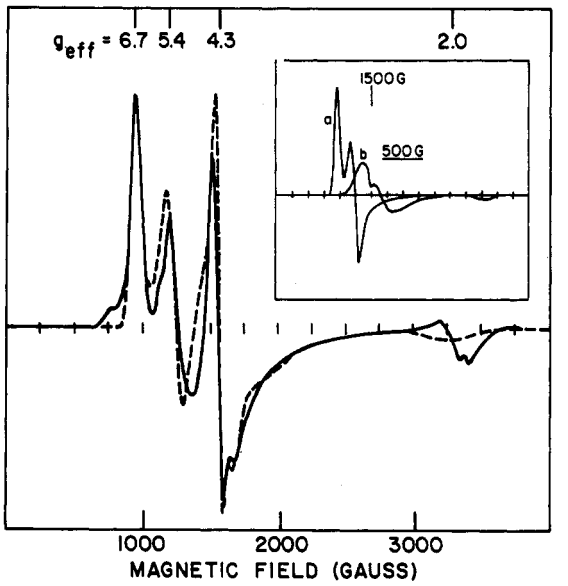

FIGURE 3: Redrawn experimental EPR spectrum for phenylalanine hydroxylase at 5 K (solid line) is compared with the spectrum (dashed line) simulated with the parameters given in Table III. The insert shows the separate signals for the major components of the simulation. Sites I and II are designated as a and b, respectively, in the insert. Instrument settings for recording the experimental spectrum were given earlier (Wallick et al., 1984; Figure 3). The specific activity of the experimental sammple is 5.0. The g_{eff} = 2 region of the experimental spectrum contains appreciable background signal, so no attempt was made to provide a simulation to fit this region.

(Gottschall et al., 1982). Other attempts to repopulate specifically a single site by the addition of substoichiometric levels of iron led to a similar distribution of iron between the two environments.

Simulation of Iron EPR Spectrum of PAH. Our previous report (Wallick et al., 1984) tentatively assigned the prominent features of EPR spectra of PAH to two different iron environments. The environment with intensity proportional to activity gives peaks at g_y and g_x = 6.7 and 5.4, while a second environment is associated with a broad peak centered around g_{eff} = 4.3 and with the fraction of inactive enzyme. To further examine this assignment, we now show a computer-simulated EPR spectrum and compare it with an experimental spectrum of PAH in Figure 3. The simulation is the weighted sum of calculations for two major iron species and two minor ones. The major contributions to the spectrum are from a lower Kramer's doublet corresponding to one iron environment and from a middle doublet with iron of different geometry. The minor components contribute sharp g = 4.3 signals. Table III lists values for the ratios of zero-field splittings (E/D = λ), effective g values, and values of D used in the simulation. Although all Kramer's doublets are populated at the temperature of the EPR measurements, the middle doublet from site I and the lower doublet from site II are not included in the simulation because by comparison they have low transition probabilities and large spectral anisotropy compared to the transitions shown in the simulation. PAH spectra at Q-band (35 GHz) show shifts of the effective g values of peaks compared to the corresponding g values at X-band (D. V. Mavrophilipos, unpublished experiments). The magnitude of these shifts is consistent with $D \simeq 1$ cm^{-1} for the component giving the g_{eff} = 6.7 and 5.4 peaks and $D < 1$ cm^{-1} for the broad feature centered around g_{eff} = 4.3. Thus, Zeeman and zero-field splitting terms in the Hamiltonian are of comparable magnitude at these EPR frequencies so that the appropriate computation solves the secular determinant exactly. The simulation program is similar to that of Aasa (1970). Transformations from expressions describing frequency-swept to field-swept EPR were made as outlined by Pilbrow (1984) and by Aasa and Vanngard (1975). The broad feature centered around g = 4.3 was simulated with a "λ-strain" model (A. S. Yang and B. J. Gaffney, submitted for publication). In the λ-strain model, the intensity of a broad feature is approximated by summing a series of spectra at closely spaced λ values, the intensities of which are normalized by a Gaussian distribution of λ values. The simulation shown in Figure 3

Table IV: Reduction of Phenylalanine-Activated Phenylalanine Hydroxylase[a]

pterin equiv per PAH subunit	pterin (μM)	obsd Tyr (μM)[b]	predicted Tyr (μM)	obsd O_2 (μM)[c,d]	predicted O_2 consumption (μM): no O_2 required for reduction	predicted O_2 consumption (μM): 0.5 O_2 required per reduction
0.5	5	3 ± 0.7	2.6	2.8 ± 0.4	2.6	5
1	10	7.2 ± 1.4	6.8	5.8 ± 0.5	6.8	10.0
1.5	15	10.7 ± 3.3	11.4	8.0 ± 1.8	11.4	15

[a] The simulations were done by assuming that all of the iron can be reduced and varying the amount of enzyme capable of turnover in order to cover the experimental range of specific activities (75–40%). The predictions shown are for enzyme of maximal specific activity because varying the specific activities did not produce significantly different results (±10%). The same rate of reduction was used for active and inactive enzyme because a single rate of reduction has been observed (Marota & Shiman, 1984). [b] The average result of three experimental runs. [c] The average result of four experimental runs. [d] Not corrected for O_2 consumption owing to pterin autooxidation, whose contribution would be within the reported error limits.

is consistent with our previous assignment of the prominent peaks to two forms of iron. In the simulation shown, for a temperature of 6 K, visible iron in site I ($\lambda = 0.032$; $D = 1$ cm^{-1}) represents 37% of the total iron while site II ($\lambda_0 = 0.20$; $\Delta\lambda = 0.05$ $D = 0.3$ cm^{-1}) plus minor components correspond to 63%. Activity assays predict that site I is 36% of the iron in this PAH sample.

Oxygen Consumption during Reduction of PAH and Phenylalanine-Activated PAH. Reduction of PAH with $6MPH_4$ will not occur in an anaerobic system (Marota & Shiman, 1984). When PAH that had not been preincubated with Phe was reduced with an equivalent of $6MPH_4$ [an equivalent of pterin per subunit of PAH is required for complete reduction under these conditions (Marota & Shiman, 1984)], 0.5 ± 0.1 equiv of O_2 was consumed per pterin equivalent. Reduction of PAH in the presence of catalase leads to O_2 consumption of 0.12 ± 0.05 equiv per pterin equivalent. Reduction of phenylalanine-activated PAH was measured in a catalytic system; the results are shown in Table IV. The predicted amount of tyrosine was calculated from the kinetic sequence:

$$PAH_{act}^{ox} + 0.5(6MPH_4) \xrightarrow{k_r} PAH_{act}^{red} + 0.5(6MPH_2)$$

$$k_r = 1.5 \times 10^7\ M^{-1}\ min^{-1}$$

$$PAH_{act}^{red} + 6MPH_4 + Phe + O_2 \xrightarrow{k_c} PAH_{act}^{red} + 6MPH_2 + Tyr + H_2O$$

$$k_c = 2.8 \times 10^7\ M^{-1}\ min^{-1}$$

The rate constants and reaction stoichiometry for the above steps were those of Marota and Shiman (1984) and Shiman (1985); no appreciable reoxidation of PAH_{act}^{red} occurs under these conditions. The good correspondence between the observed and predicted Tyr levels (Table IV, columns 3 and 4) serves as a control and confirms reduction of the bulk of the enzyme pool in the initial step. The values of O_2 consumption thus were measured under appropriate conditions and are compared to values predicted on the basis of no oxygen or 0.5 equiv of oxygen required per reduction (Table IV, columns 5–7). The observed oxygen consumption is closer to the value predicted for reduction of PAH_{act}^{ox} without oxygen consumption than if reduction required oxygen.

Activity Retention during PAH Reduction. Reduction of PAH with an equivalent of $6MPH_4$ per mole of PAH subunits leads to a loss of hydroxylase activity as shown in Table V. The activity loss was complete by the time the initial activity measurement after reduction was made. Hydroxylase activity is unaltered when reduction with an equivalent of pterin occurs in the presence of 32 μg/mL catalase.

EPR Spectra of Reduced Phenylalanine Hydroxylase. The EPR spectra obtained upon reduction of the enzyme with slightly more then a equivalent of dithionite and its subsequent

Table V: Activity Retention during Phenylalanine Hydroxylase Reduction

pterin equiv per PAH subunit	catalase (μg/mL)	activity (units/mL) before reduction	activity (units/mL) after reduction	activity after reduction/activity before reduction
1	0	36, 36	24, 24	0.67, 0.67
1	32	36, 36	37, 39	1.0, 1.1

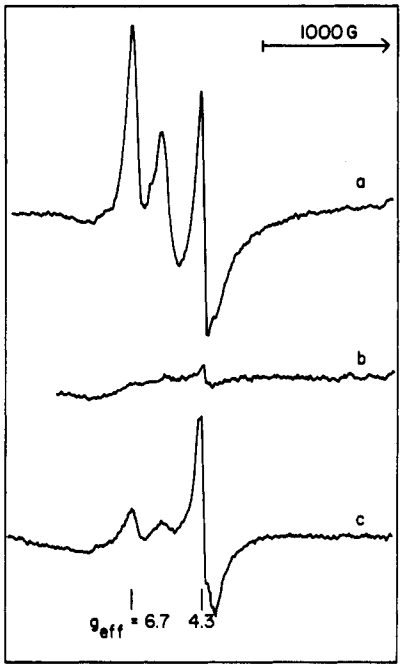

FIGURE 4: Reduction of PAH with dithionite. (a) Paramagnetic resonance spectrum of PAH (190 μM, sp act. = 5.6). Instrument settings are those given in Figure 1a except that microwave power is 0.05 mW. (b) EPR spectrum of PAH (190 μM, sp act. = 0.4) in the presence of excess dithionite. The instrument settings are described in (a). (c) EPR spectrum of PAH (190 μM, sp act. = 0.6) described in (b) after exposure to air. Instrumental settings are described in (a). All spectra were recorded at 5 K.

reoxidation are given in spectra b and c of Figures 4, respectively. Comparson of Figure 4b with the holoenzyme spectrum in Figure 4a shows that excess dithionite reduces the iron in both environments. After the enzyme was exposed to air, the reoxidized spectrum was obtained. Activity recovery was 10% of the initial activity.

Titration of PAH with Inhibitors. The cofactor analogue 5-thiapterin, a competitive inhibitor against the pterin cofactor, and *o*-phenanthroline were added to PAH. Both compounds, like Phe, led to a loss of signal intensity at $g_{eff} = 6.7$ and 5.4 that was compensated by a corresponding increase in signal intensity centered around $g_{eff} = 4.3$ (data not shown).

Dopamine and epinephrine are known inhibitors of the PAH reduction step (Marota & Shiman, 1984). The EPR spectra

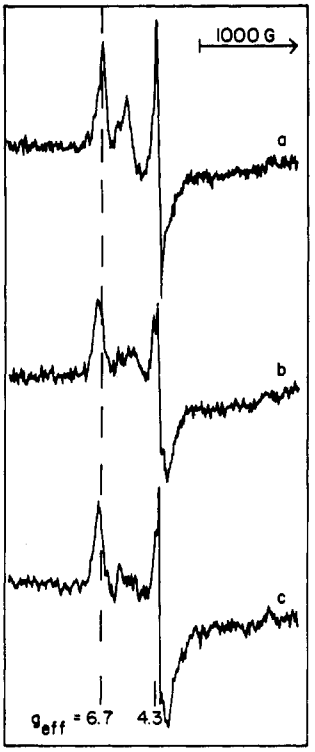

FIGURE 5: EPR spectra at 5 K of PAH in the presence of catecholamines. (a) Paramagnetic resonance spectrum of PAH (58 μM, sp act. = 3.4) at 5 K. Instrument settings are as in Figure 1a except that receiver gain = 4×10^4. (b) EPR spectrum of PAH (46 μM) in the presence of 80 μM dopamine. Instrument settings are described in (a). (c) EPR spectrum of PAH (53 μM) in the presence of 0.9 mM epinephrine. Instrument settings are described in (a).

of PAH in the presence of saturating amounts of dopamine and epinephrine are shown in spectra b and c of Figures 5, respectively. In contrast to the large EPR changes noted above, the catecholamine inhibitors of reduction give only a small downfield shift in the low-field maximum from g_{eff} = 6.7 to $g_{eff} \sim 7.0$ with no significant loss in intensity. The signal in the g_{eff} = 4.3 region is also slightly altered by the addition of the catecholamines.

Discussion

The specific activity of PAH in crude homogenates was determined in order to establish whether the two iron environments were populated before purification so as to exclude the possibility that inactive enzyme was generated during the purification procedure. Crude hydroxylase averages less than 50% of maximal specific activity even after iron activation. Since there is an inverse correlation between PAH specific activity and the iron distribution between two environments, one of which is characteristic of inactive enzyme, it appears that crude hydroxylase is a mixture of subunits with iron in both environments.

The objective of the reconstitution experiments was to determine whether the two iron sites readily interconvert. The experiments depicted in Figure 1 illustrate selective removal of the iron from the g_{eff} = 6.7 and 5.4 region without reequilibration owing to the remaining iron at g_{eff} = 4.3. Thus, there is no exchange between the two sites. Similarly, the experiment shown in Figure 2 demonstrates that addition of iron to iron-free enzyme repopulates both sites. Thus, the incorrect incorporation of iron as a consequence of iron activation of the crude homogenate is eliminated as an explanation of the g_{eff} = 4.3 signal. Clearly, the two environments are fixed in the purified as well as in the crude enzyme.

An obvious question is whether one of the two iron sites is more prone to the obligatory reduction. At 0.5 equiv of $6MPH_4$ relative to enzyme subunits, the signals at g_{eff} = 6.7 and 5.4 are significantly diminished relative to g_{eff} = 4.3 [Figure 1 in Benkovic et al. (1985a)]. Since the enzyme does not significantly reoxidize during the time of observation (Marota, 1983), it would appear that the active site iron is more susceptible to reduction. At higher levels of reductant (both pterin and dithionite), the signals associated with both iron environments are deleted (data not shown for reduction with pterin).

It is now clear that the site of reduction is the Fe^{3+} center. However, the reduction has several puzzling aspects. First, the reduction of PAH by $6MPH_4$ does not proceed to a significant extent anaerobically (Marota & Shiman, 1984). Second, the stoichiometry of the reduction of PAH with $6MPH_4$ depends on whether *o*-phenanthroline or Phe is present during the reduction reaction. The stoichiometry is 0.5:1 ($6MPH_4$:PAH subunit) or 1 e^-/subunit if the chelator or activator is present but 1:1 or 2 e^-/subunit if it is absent (Wallick et al., 1984; Marota & Shiman, 1984; Shiman, 1985). Third, strong anaerobic reducing agents such as dithionite reduce PAH with a 1 e^-/subunit stoichiometry (Wallick et al., 1984).

Our hypothesis that these different reduction stoichiometries are related to the presence of Phe is substantiated by the results in Table IV. The findings are consistent with the absence of O_2 consumption in the reduction step for phenylalanine activated enzyme but oxygen uptake amounting to 0.5 O_2/subunit for the non-phenylalanine-activated enzyme. The reduction in the absence of Phe can be formulated as

$$Fe^{3+}\text{–PAH} + 6MPH_4 + O_2 \rightarrow Fe^{2+}\text{–PAH} + O_2^{\bullet -} + 6MPH_2 \quad (1)$$

$$2O_2^{\bullet -} \rightarrow O_2 + HOOH \quad (2)$$

The presence of catalase should lower the oxygen consumption from 0.5 equiv per pterin equivalent to 0.25 equiv per pterin equivalent (eq 3). The observed oxygen consumption is actually 0.12 equiv per pterin equivalent, which we attribute to additional hydrogen peroxide formed during autooxidation of the pterin.

$$2HOOH \xrightarrow{\text{catalase}} O_2 + 2H_2O \quad (3)$$

The presence of Fe^{2+}–PAH and hydrogen peroxide in solution could give rise to Fenton's chemistry (eq 4) (Dix & Benkovic, 1985). Several attempts to trap hydroxide radical were unsuccessful, not unexpectedly because the enzyme is available as a trap. The reaction of hydroxide radical with the protein may be responsible for the loss of about 40% of the enzyme's activity in the absence of catalase. Catalase alone is sufficient to protect PAH from loss of activity during reduction. Oxygen is proposed to capture any semiquinone radical species derived from $6MPH_4$ during 1-e^- transfer to the Fe^{3+} center. In the presence of Phe and *o*-phenanthroline, the pterin reduction is coupled to *two* Fe^{3+} centers although at present there is no evidence for antiferromagnetic coupling between those centers.

$$Fe^{2+}\text{–PAH} + HOOH \rightarrow Fe^{3+}\text{–PAH} + OH^- + OH^{\bullet} \quad (4)$$

In examining EPR spectra in the presence of ligands, we find that the ligands can be placed in three categories on the basis of the changes they cause in the spectra. Phe, 5-thiapterin, and *o*-phenanthroline cause a large shift in the iron environment associated with catalytic activity from slight

rhombic distortion to nearly maximum rhombicity. We previously reported that high concentrations of glycerol give the same spectral change (Wallick et al., 1984). The catecholamines represent a second category of ligands that bind at or near the Fe^{3+} site but cause only a slight decrease in rhombic distortion. Tryptophan is in the third category; it binds at the active site of the enzyme but produces no spectral change (data not shown).

The observation that three ligands, phenylalanine, 5-thiapterin, and *o*-phenanthroline, alter the low-field signal so that it now is shifted to the region centered around $g_{eff} = 4.3$ may be interpreted with respect to the changes in reduction stoichiometry and oxygen consumption in the presence of phenylalanine. We suggest that phenylalanine binding at a distinct activation site (Shiman, 1980) alters the iron environment to allow reduction without oxygen consumption. Coupling of the irons through a decrease in their separation distance in the presence of phenylalanine would be a reasonable explanation, but such a proximity is not revealed in any unusual temperature dependence of EPR intensity. The fact that hydroxylase reduction by pterin in the presence of *o*-phenanthroline also requires 0.5 equiv of pterin for complete reduction suggests that *o*-phenanthroline may have brought about the same change in the iron environment as Phe. Reduction stoichiometry has not been determined in the presence of 5-thiapterin, which also effects this change.

Inhibition by catecholamines is known to be at the reductive step, but hydroxylase turnover is not altered (Marota & Shiman, 1984). A possible interpretation of the EPR results with catecholamines is that they displace one or more of the ligands that the protein provides to iron. Tryptophan, a substrate that binds to the catalytic site but does not bind to the activation site, does not alter the EPR spectrum. This result, together with the phenylalanine activation studies (Benkovic et al., 1985b), suggests that a substrate bound in the catalytic site neither coordinates directly to iron nor causes changes in the geometry of its ligand field.

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Registry No. PAH, 9029-73-6; $6MPH_4$, 942-41-6; L-Phe, 63-91-2; Fe, 7439-89-6; 5-thiapterin, 84099-73-0; *o*-phenanthroline, 66-71-7; dithionite, 14844-07-6; dopamine, 51-61-6; epinephrine, 51-43-4.

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