Rat Liver Phenylalanine Hydroxylase, an Iron Enzyme

(Received for publication, April 6, 1972)

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SUMMARY

Phenylalanine hydroxylase that is essentially pure contains 1 to 2 moles of iron per mole of enzyme (assuming a molecular weight of 100,000). Electron spin resonance (ESR) studies have shown that the iron is present in the high spin ferric form. The addition of substrates (i.e. phenylalanine and dimethyltetrahydropterin) causes the signal for this form to disappear. The iron, 50 to 80%, can be removed by treatment of sucrose gradient purified enzyme with o-phenanthroline and cysteine; the loss of iron leads to the loss of enzymatic activity. The enzyme activity is restored by FeCl₃ with half-maximum restoration achieved at 5 × 10⁻⁶ M FeCl₃. Mercurocyan, nickelous, cobalt, manganese, cupric, chromium, cadmium, and zinc ions are ineffective in restoring activity to the apoenzyme (o-phenanthroline-treated enzyme).

As judged by gel electrophoresis, the apoenzyme has the same charge and molecular weight as the holoenzyme. The apoenzyme, however, is much less stable at 25° than the holoenzyme; FeCl₃ restored the original stability to the apoenzyme. Furthermore, the apoenzyme has only three free cysteines per 50,000 molecular weight subunit whereas the holoenzyme has five cysteines per subunit. The amino acid composition of the enzyme was also determined.

Phenylalanine hydroxylase was assayed in a reaction mixture containing the following components (in micromoles) in a final volume of 1.0 ml: potassium phosphate, pH 6.8, 100; L-phenylalanine, 2.0; TPN⁺, 0.25; glucose, 250; glucose dehydrogenase, 300 mg; dihydropteridine reductase, 100 μg; tetrahydropterin and hydroxylase, in concentrations indicated in the legends. The reaction mixture was routinely incubated 30 min at 25° and stopped by addition of 1 ml of 1 N perchloric acid. Tyrosine produced was determined fluorometrically by the nitrosonaphthol method (5). The enzyme was also assayed spectrophotometrically by measurement of the phenylalanine dependent oxidation of TPNH (1).

To prepare the protein for iron determinations, it was wet-ashed with sulfuric and nitric acid, followed by perchloric acid. The iron content of the ashed protein was determined colorimetrically; the complex of reduced iron with bathophenanthroline, was extracted into isomyl alcohol and its absorbance at 535 nm was determined (6). For copper analysis, the enzyme was ashed in the same manner; the copper-bathocuproine complex was formed and its absorbance at 480 nm was determined (6). Protein was determined by the method of Lowry (7).

Sucrose gradients were made with a Beckman sucrose gradient former. The light syringes contained 0.01 M Tris-HCl, pH 8.0, and the heavy syringes contained 28% (w/v) sucrose buffered with 0.01 M Tris-HCl, pH 8.0. Centrifugation was performed at 21,000 rpm in a Beckman L2-65B ultracentrifuge in a SW 65 rotor.

The enzyme samples for amino acid analysis were peak fractions from the G-200 Sephadex step (2), which were further purified by sucrose gradient centrifugation. The peak tubes from the sucrose gradient were freed of salts and sucrose by pressure dialysis and the protein was lyophilized in a combustion tube. The samples were hydrolyzed for the indicated time with 5.7 N constant boiling HCl at 105° under a vacuum. The amino acid analyses were performed with a Hitachi Perkin-Elmer KLA-3B amino acid analyzer equipped with an autosampler utilizing a zinc-ligand system. To determine the total cystine and cysteine, the enzyme was oxidized with performic acid (8). The performic acid was formed by mixing hydrogen peroxide (30%) and formic acid (88%) in a 1:10 ratio. The reaction was carried out for 16 hours at 0° and excess performic acid was removed by lyophilization. Cysteine was estimated by reaction with 3,3'-dithiobis(6-nitrobenzoic acid) (9), or by reaction with N-ethyl(6-mercaptoacetoxyethyl)maleimide (1.7 mg per mole, Schwarz-Mann, Orangeburg, New York) at pH 7.0. The unreacted N-ethyl(6-mercaptoacetoxyethyl)maleimide was removed by chromatography on G-25 Sephadex.

MATERIALS

TPNH and crystalline catalase were obtained from Boehringer Mannheim Corp. Dihydropteridine reductase was obtained from sheep liver and purified through the calcium phosphate gel step (1). Tetrahydrobiopterin was a gift from Dr. Long, Hoffmann-La Roche. Bathophenanthroline was obtained from Frederick Smith Chemical Co., Columbus, Ohio. Glucose dehydrogenase was prepared from beef liver (4) as previously described.
The tryptophan content of phenylalanine hydroxylase was determined from the absorbance of the protein at 280 nm and 294 nm in 0.1 N NaOH and from the tyrosine content determined by amino acid analysis (10). Tryptophan was also measured colorimetrically by treatment of the intact protein with the reagent of Spies and Chambers (11).

Electron spin resonance studies were carried out with a Varian V-4500 X band spectrometer. A 9-inch magnet was used. The field modulation was 100 kHz and was regulated by a Fieldial. The microwave frequency was measured by a cavity wave meter. The applied magnetic field, H, was calibrated by using a proton resonance line. The sample was placed in a quartz tube having a 2.5 mm I.D. in a Joel cylindrical cavity. The modulation amplitude was 10 gauss.

The hydroxylase (50 pg of 70% pure enzyme) was also assayed in the absence of the enzymatic reductase, that was being inhibited. To prove this point, the hydroxylase was assayed in the absence of the enzymatic-regenerating system (i.e. TPNH and dihydropteridine reductase), it was important to show that it was the hydroxylase, rather than the reductase, that was being inhibited. To prove this point, the hydroxylase was assayed in the absence of the enzymatic-regenerating system by direct measurement of the phenylalanine-dependent oxidation of the tetrahydropterin (12). It was found that under these conditions, the extent of inhibition by o-phenanthroline and diethyldithiocarbamate was exactly the same as was previously found in the coupled assay system. Therefore, the inhibition by chelators illustrated in Table I is most likely due to inhibition of the hydroxylase.

**Table I**

| Chelator          | Concentration | Inhibition | Pterin cofactor used |
|-------------------|---------------|------------|----------------------|
| 2,2'-Dipyridine   | 1 x 10^{-3}   | 34         | Tetrahydrobiopterin  |
| 2,2'-Dipyridine   | 2.5 x 10^{-4} | 77         | Tetrahydrobiopterin  |
| o-Phenanthroline  | 1.0 x 10^{-5} | 40         | 7-Methyltetrahydropterin |
| o-Phenanthroline  | 2.0 x 10^{-5} | 75         | 7-Methyltetrahydropterin |
| m-Phenanthroline  | 2.0 x 10^{-5} | 83         | 7-Methyltetrahydropterin |
| o-Phenanthroline  | 5.0 x 10^{-5} | 100        | Tetrahydrobiopterin  |
| 8-Hydroxyquinoline| 1.0 x 10^{-5} | 0          | 7-Methyltetrahydropterin |
| 8-Hydroxyquinoline| 2.0 x 10^{-5} | 67         | 7-Methyltetrahydropterin |
| Diethyldithiocarbamate | 1.0 x 10^{-2} | 100        | 7-Methyltetrahydropterin |

Metal-analysis—Since the foregoing evidence indicated that phenylalanine hydroxylase is a metallo-enzyme, and earlier evidence indicated that the metal might be iron (1), the purified enzyme was analyzed for iron. Our method for purifying the hydroxylase yields enzyme which is 90% pure by several criteria (2). Sucrose gradient centrifugation of this material yielded a single peak of protein and activity. Iron analyses of fractions from the sucrose gradient demonstrated that a peak of iron corresponded with the activity and protein peak (Fig. 1). The spectrum of the complex of the bathophenanthroline and the metal from the enzyme was nearly identical to the complex of bathophenanthroline and iron (Fig. 2). Five determinations of the iron content of this sucrose gradient purified enzyme gave a mean of 1.4 (range 0.7 to 2.0) g atoms of iron per mole of 100,000$^1$

$^1$ Previous studies with sucrose gradient centrifugation and disc-gel electrophoresis with different percentage acrylamide gels indicated the molecular weight of the hydroxylase dimer is 110,000. Sodium dodecyl sulfate electrophoresis demonstrated that molecular weight of the monomer is 51,000 which would give a dimer molecular weight of 102,000 (2). Recently, sedimentation equi-
was the o-phenanthroline reaction with an aliquot of dialysate from enzyme equal to the volume of enzyme analyzed. The blank consisted of a dialysate from enzyme, ashed, and reacted with bathophenanthroline. The blank was the o-phenanthroline reaction with an aliquot of dialysate from enzyme equal to the volume of enzyme analyzed.

**Table II**  
**Activation of phenylalanine hydroxylase by FeCl₂ and DTT**

| Time of addition | Enzyme activity | mmoles tyrosine/min/mg |
|-----------------|-----------------|------------------------|
| 0 min           |                 |                        |
| FeCl₂           | 56              |                        |
| FeCl₂ + DTT     | 144             |                        |
| DTT             | FeCl₂ + DTT    | 56                     |
| DTT             | FeCl₂          | 76                     |

The hydroxylase (70% pure) was previously incubated in the complete reaction mixture without phenylalanine and tetrahydrobiopterin for 8 min and the reaction was started by addition of phenylalanine (1 mM, final concentration) and tetrahydrobiopterin (0.01 mM). FeCl₂ (2 mM) and DTT (1 mM) were added at indicated times.

molecular weight phenylalanine hydroxylase. This wide range in values might have resulted from variable degrees of loss of iron during purification of the hydroxylase. Consistent with this postulate is the finding that the activity of the hydroxylase can be increased by incubation of the enzyme with FeCl₂ and dithiothreitol (Table II). Under these conditions, FeCl₂ activated the hydroxylase 50 to 100%. One preparation of hydroxylase which had 1.1 g atoms of iron per 100,000 molecular weight, was activated 80% by treatment with FeCl₂ and DTT. This was the degree of activation expected if the fully active enzyme contained 2.0 g atoms of iron per 100,000. This amount of iron would correspond to 1 iron per 50,000 molecular weight subunit of the enzyme. It is of interest that when the FeCl₂ and DTT were added at the beginning of the reaction in the presence of the substrates, no activation was seen. Furthermore, the added iron does not form a tight complex with the enzyme because precipitation of the enzyme-iron mixture with ammonium sulfate led to the recovery of enzyme with the same low activity as the untreated enzyme.

A second peak of iron appeared at the bottom of the gradient when the 90% pure enzyme was centrifuged for a shorter period (60,000 rpm for 1 hour). The faster migrating iron peak had a sedimentation coefficient of 70 S and migrated nearly the same as ferritin. That the major impurity in 90% pure hydroxylase is ferritin is also indicated by the finding that its molecular weight and absorption spectrum are essentially the same as ferritin. This impurity can be conveniently separated from the hydroxylase by sucrose gradient centrifugation because its high iron content gives it a very high rate of sedimentation. All subsequent studies were carried out with phenylalanine hydroxylase which had been purified to greater than 95% by sucrose gradient centrifugation.

An absorbance spectrum of this purified preparation of the hydroxylase (Fig. 3) revealed a peak at 280 nm and a 280 nm to 260 nm ratio of 1.6. There also was a small shoulder at 400 nm. This type of spectrum is often seen with non-heme iron proteins, such as pyrocatechase (13) and transferrin (14). It should be noted that the spectrum obtained for purified phenylalanine hydroxylase is different from those seen for non-heme iron proteins containing acid-labile sulfur (15). These latter proteins exhibit absorption peaks at 315 to 335 nm, 410 to 420 nm, and 450 to 460 nm. Since several other hydroxylases have been shown to require copper for enzymatic activity (16, 17), purified phenylalanine hydroxylase was analyzed for copper. This analysis revealed that there are only 0.2 g atoms of copper per mole of 100,000 molecular weight hydroxylase.

**ESR Studies**—As another means of characterizing the metal in the enzyme, sucrose gradient purified phenylalanine hydroxylase was analyzed by ESR spectroscopy. A dramatic signal was observed at \( g = 4.23 \) (Fig. 4) for the enzyme in 0.02 M Tris-HCl buffer, pH 6.8, in the presence of oxygen. A signal at this \( g \) value is characteristic of a high-spin ferrie ion (18). When phenylalanine and 6,7-dimethyltetrahydropterin, the other two substrates for the enzyme, were added, 90% of the signal at \( g = 4.23 \) disappeared (Fig. 4). This disappearance of the \( g = 4.23 \) signal could be due either to a reduction of the iron to the ferrous form, or a change from high to low spin state of Fe²⁺ due to a change in its ligand field. Forty percent of the signal reappeared when the reaction mixture was bubbled with oxygen. After these ESR measurements had been completed, the enzyme
weight and charge of the hydroxylase are not affected by the patterns in disc gel electrophoresis, indicating that the molecular absence of iron. The apoenzyme, however, was much more completely inactivated the apoenzyme. The apo- and holoenzyme gave identical enzyme were compared to see if the iron plays a structural role labile to incubation at 25° than the holoenzyme (Fig. 6). The apoenzyme had a half-life of less than 1 min at 25°, whereas the holoenzyme’s half-life was greater than 20 min. Addition of

Preparation of Apoenzyme—The above studies demonstrated that iron is intimately associated with phenylalanine hydroxylase and that the iron may be involved in the catalytic activity of the enzyme. Additional support for the idea that iron is an essential component of phenylalanine hydroxylase, was obtained by removal of most of the iron from the hydroxylase. By treatment of the sucrose gradient purified enzyme with o-phenanthroline and cysteine for 4 hours at 0°, it was possible to remove 50 to 80% of the iron (Table III). In some experiments (see Experiment 3, Table III) the enzyme activity decrease to a greater extent than did the iron content, an indication that some o-phenanthroline-iron complex might remain bound to the enzyme. An absorbance spectrum of the apoenzyme even after four ammonium sulfate precipitations revealed that some o-phenanthroline-iron complex might remain bound to the enzyme. Addition of FeCl₂ back to this apoenzyme gave nearly full restoration of enzyme activity. Saturation with FeCl₂ restored phenylalanine hydroxylase activity with hyperbolic kinetics. A plot of the reciprocal of percentage control activity as a function of the reciprocal of the FeCl₂ concentration revealed that 5 \times 10^{-5} \text{ M} \text{ FeCl}_2 gave maximum restoration of enzyme activity (Fig. 5). When a variety of metals were tested for their ability to restore the activity of the apoenzyme, only ferrous ions were effective (Table IV). As can be seen, mercuro and cupric ions completely inactivated the apoenzyme.

Some of the physical characteristics of the apo- and holoenzyme were compared to see if the iron plays a structural role in the hydroxylase. The apo- and holoenzyme gave identical patterns in disc gel electrophoresis, indicating that the molecular weight and charge of the hydroxylase are not affected by the absence of iron. The apoenzyme, however, was much more labile to incubation at 25° than the holoenzyme (Fig. 6). The apoenzyme had a half-life of less than 1 min at 25°, whereas the holoenzyme’s half-life was greater than 20 min. Addition of

Sucrose gradient purified hydroxylase (1 to 1.5 mg) was incubated for 4 hours at 0° in 20 mM cysteine buffered to pH 6.8 with 0.1 M Tris-HCl with or without 3.3 mM o-phenanthroline. The iron-o-phenanthroline complex was removed from the enzyme by four successive precipitations with 40% saturated (w/v) ammonium sulfate. A portion of the resulting enzyme was assayed for enzyme activity with and without 2 \text{ mM} \text{ FeCl}_2. The remaining enzyme was assayed for iron content by the bathophenanthroline method (see “Methods”). The assay contained 1.0 mM phenylalanine and 0.015 mM tetrahydrobiopterin. The order of addition of the last three components added to the assay was FeCl₂, phenylalanine, and tetrahydrobiopterin.
TABLE IV
Effect of various metal ions on o-phenanthroline extracted phenylalanine hydroxylase

Fifty percent pure hydroxylase was extracted with o-phenanthroline as described in Table III. All the metals except FeCl₂ were tested at a final concentration of 4 mM. FeCl₂ was tested at 2 mM. The assay contained 2.0 mM phenylalanine and 0.016 mM tetrahydrobiopterin.

| Prior treatment | Additions to assay | Enzyme activity |
|-----------------|--------------------|-----------------|
|                 |                    | nmoles tyrosine/ min/mg |
| None            | FeCl₂              | 76              |
| None            | CoCl₂              | 10              |
| 0-Phenanthroline | FeCl₂         | 65              |
| 0-Phenanthroline | HgCl₂         | 0               |
| 0-Phenanthroline | MnCl₂             | 7               |
| 0-Phenanthroline | CaCl₂             | 8               |
| 0-Phenanthroline | NiCl₂             | 5               |
| 0-Phenanthroline | ZnSO₄             | 9               |
| 0-Phenanthroline | CuCl₂             | 0               |

FIG. 6. Effect of preliminary incubation on activity of apoenzyme versus holoenzyme. Apoenzyme (o-phenanthroline extracted) and holoenzyme (40% pure) were previously incubated indicated times at 25° with or without 2 mM FeCl₂ or 2 mM phenylalanine. All samples were assayed in the presence of 2 mM FeCl₂. The control for the holoenzyme was not previously incubated and had an activity of 45 nmoles per min per mg. The apoenzyme control had an activity of 50 nmoles per min per mg. The tetrahydrobiopterin concentration was 0.009 mM.

subunit, whereas the apoenzyme had only 2.9 cysteines. Apparently two of the apoenzyme's cysteines oxidize readily. The redusing agent DTT, however, did not prevent the loss of activity during incubation of the apoenzyme at 25°. Thus, the oxidation of the two cysteines does not explain the increased lability of the apoenzyme.

Amino Acid Composition—The amino acid composition of the sucrose-gradient purified hydroxylase was analyzed (Table V). Ninety-two percent of the applied protein nitrogen was recovered in the amino acids. From the nitrogen analysis and amino acid composition the mass of the protein was calculated. Using this estimate of the protein concentration it was found that 1 mg per ml of the hydroxylase has an absorbance of 0.95 at 280 nm. Since there were 5 cysteines and 7 cystic acids per 50,000 molecular weight subunit, there must be one disulfide per subunit. It should be noted that the amino acid composition is an average of the composition for two subunits of the same molecular weight (by SDS gel electrophoresis) but slightly different charge (by urea gel electrophoresis). From the amino acid composition, a specific volume of 0.72 was calculated.

TABLE V
Amino acid analysis of phenylalanine hydroxylase

| Amino acid | Hydrolysis time | Residues per mol(1) | Integral residues |
|-----------|-----------------|---------------------|------------------|
|           | 24 hrs          | 72 hrs              | average          |
| Aspartic  | 1.41            | 1.38                | 1.40             | 71.3 | 71 |
| Threonine | 0.38            | 0.37                | 0.38             | 20   | 20 |
| Serine    | 0.56            | 0.51                | 0.54             | 29.9 | 30 |
| Glutamic | 1.00            | 1.04                | 1.07             | 56.3 | 56 |
| Proline   | 0.49            | 0.45                | 0.47             | 26.2 | 26 |
| Alanine   | 0.50            | 0.52                | 0.55             | 31.2 | 31 |
| Valine    | 0.30            | 0.41                | 0.41             | 20.9 | 21 |
| Methionine| 0.064           | 0.064               | 0.06             | 3.6  | 4  |
| Isoleucine| 0.30            | 0.30                | 0.30             | 19.3 | 19 |
| Leucine   | 0.89            | 0.79                | 0.84             | 46.2 | 46 |
| Tyrosine  | 0.28            | 0.28                | 0.28             | 13.8 | 14 |
| Phenylalanine | 0.41 | 0.41                | 0.41             | 20.9 | 21 |
| Lysine    | 0.45            | 0.45                | 0.45             | 22.5 | 23 |
| Arginine  | 0.33            | 0.41                | 0.38             | 20.8 | 21 |
| Histidine | 0.15            | 0.16                | 0.16             | 8.3  | 8  |
| Cysteic acid | 0.15          | 0.13                | 0.13             | 6.8  | 7  |
| Cysteine(2) | 0.47         | 0.5                 | 0.5              | 4.7  | 5  |
| Tryptophan| 0.24            | 0.24                | 0.24             | 2.2  | 2  |

(1) The molecular weight utilized is 50,000.
(2) Values were obtained by extrapolation to zero hydrolysis time.
(3) Highest values are used.
(4) Cysteine was determined by titration with DTNB and [14C]-NEM.
(5) Tryptophan was determined spectrophotometrically and with p-dimethylaminobenzaldehyde.

molecular weight subunit, there must be one disulfide per subunit. It should be noted that the amino acid composition is an average of the composition for two subunits of the same molecular weight (by SDS gel electrophoresis) but slightly different charge (by urea gel electrophoresis). From the amino acid composition, a specific volume of 0.72 was calculated.

Discussion

These results demonstrate that the sucrose gradient purified phenylalanine hydroxylase, which is about 95% pure, contains between 1 and 2 moles of iron per mole of enzyme (assuming 100,000 molecular weight). Since the subunit molecular weight of the hydroxylase is 50,000 (2), it seems likely that there is 1 iron per subunit. Iron values lower than 1 might be due to the loss of some of the iron during purification of the enzyme; this possibility is supported by our observations that incubation of the enzyme with FeCl₂ and DTT activates the enzyme 50 to 100%.

ESR studies revealed that the purified hydroxylase gives a signal at g = 4.23. A signal at g = 4.23 is attributed to a high spin ferric ion surrounded by an environment having a large component of rhombic symmetry (18). The high spin state of the d electrons of the ferric ion results from a small degree of splitting of the two groups of 3d orbitals. It is likely that this small degree of splitting is due to a greater ionic than covalent, binding between the iron and its ligands (18). Some of the iron might be present in another form giving a weaker signal. Since addition of the substrates caused the g = 4.23 signal to disappear,

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we have concluded that this iron is involved in the enzymatic catalysis. The fact that removal of iron correlated with a loss of enzymatic activity further supports the postulate that the iron plays an important role in the hydroxylation reaction. Furthermore, since all metals ions tested, only iron restored the activity to the apoenzyme, the metal requirement is highly specific. This finding contrasts with the report that Pseudomonas phenylalanine hydroxylase is activated by mercuric, cadmium, cupric, and cuprous ions, as well as ferrous ions (19).

Although the present studies have established for the first time that a pterin-dependent hydroxylase is an iron enzyme, and that the iron is essential for the enzyme's catalytic activity, the precise mechanism by which the enzyme-bound metal participates in the reaction is not clear.

From previous studies on the role of metals in oxygenase-catalyzed reactions, it is apparent that dioxygenases require metals (20), but that not all monoxygenases do. In particular, a large group of FAD-dependent monoxygenases have been shown to contain neither iron nor copper (20). On the other hand, there have been many reports of monoxygenases, such as 2,5-diketocamphane-lactonizing enzyme (21), fatty acid CoA-desaturating enzyme (22), p-hydroxybenzoate hydroxylase (23), and bovine adrenal tyrosine hydroxylase (24), requiring Fe^3+ for full activity. It should be noted that with the last enzyme, it has been shown that the reduced enzyme is an intermediate in the reaction is the finding that the mechanism of Fe^3+ to decompose H_2O_2, and thereby protect the hydroxylase from inactivation by endogenously-generated peroxide (25). With that enzyme, catechol can substitute for Fe^2+ (25).

Two quite distinct roles can be envisioned for the iron in phenylalanine hydroxylase: it could be involved in maintaining the structure of the active enzyme (e.g. by holding subunits together (20)), or it could be directly involved in the catalytic mechanism.

There is no evidence in favor of the idea that the function of iron in the hydroxylase is merely a structural one; the apoenzyme and the holoenzyme do not differ markedly in either molecular weight or net charge.

If the metal is involved in the catalytic functioning of the enzyme, it could take part in the activation of oxygen, or, less likely, in the activation of phenylalanine or the tetrahydropterin. If it participates as oxygen activation, the metal could facilitate the transfer of electrons between the metal and oxygen. Such a role for the metal has been suggested for the copper-containing monoxygenase, dopamine beta-hydroxylase, where it has been shown that the enzyme-bound copper undergoes a cycle of reduction (ascorbate serving as electron donor) and re-oxidation during the hydroxylation reaction (16, 27). Consistent with the demonstration that the reduced copper is an intermediate in the reaction is the finding that the mechanism of that hydroxylase is of the ping-pong type (28).

In contrast to the findings with dopamine beta-hydroxylase, recent studies have revealed that all three substrates must be bound to phenylalanine hydroxylase before any detectable reaction occurs. Stoichiometric enzyme studies have demonstrated that there is no reduction of the enzyme in the absence of both the other two substrates. Kinetic studies have shown that the phenylalanine hydroxylase mechanism is of the sequential, rather than ping-pong, type. The disappearance of the g = 4.23 ESR signal of phenylalanine hydroxylase on addition of DMPIH and phenylalanine (Fig. 4) is consistent with reduction of Fe^2+ to Fe^3+. In light of the kinetic and stoichiometric enzyme studies, however, this reduction of iron may not be a distinct step, but a transient event occurring during a concerted mechanism. Such a mechanism would be in accord with Hamilton's proposal that iron might form a link for the flow of electrons from the tetrahydropterin to oxygen (29). Alternatively, the disappearance of the g = 4.23 signal upon addition of the substrates may be due to a change of the Fe^3+ from a high to a low spin state caused by an alteration of the metal's ligand field. In this regard, it should be noted that dioxygenase, pyrocatechase, also has an ESR signal characteristic of high spin Fe^3+ (g = 4.28). With this enzyme, too, the signal disappears during catalysis, but this change is not believed to be due to reduction of the iron (13).

Studies with model hydroxylation systems have suggested that iron might be more directly involved in the activation of oxygen. Oxotransition metals such as ferrate and manganate ions can catalyze the hydroxylation of olefins (30). Manganate ions, furthermore, promote the hydroxylation of aromatic compounds resulting in the NIH shift characteristic of the phenylalanine hydroxylase reaction (31). These findings have suggested that an oxoiron (Fe = 0) species might be the active oxidant in hydroxylation reactions (30). Further studies will be required to elucidate the role of the iron in the phenylalanine hydroxylase-catalyzed reaction.

Acknowledgments—We thank Dr. Hideo Kon for performing the ESR studies and Dr. Jules Gladner for performing the amino acid analysis.

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