Blewitt et al., 1985). The results also imply that ApUp bound to toxin within an endosome would probably not prevent pH-induced membrane insertion and subsequent steps in the intoxication process. Under our experimental conditions, we did not observe a rapid release of DT from cell surface receptors, which is consistent with the insertional model.

Eidels and co-workers reported that under certain experimental conditions the nucleotide-bound form of diphtheria toxin was several hundredfold less toxic than the nucleotide-free form of the toxin (Proia et al., 1981). They attributed this decrease in cytotoxic potential to the inability of the nucleotide-bound form of the toxin to interact with cell surface receptors. Our observation that the ApUp binding site on receptor-bound toxin is accessible and not greatly perturbed conflicts with this conclusion. In a separate communication we report results on the effect of dinucleotides, including ApUp, on the cytotoxic action of DT.

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Registry No. ApUp, 1985-21-3.

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Phenylalanine Hydroxylase from *Chromobacterium violaceum* Is a Copper-Containing Monooxygenase. Kinetics of the Reductive Activation of the Enzyme†

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ABSTRACT: Pterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* contains a stoichiometric amount of copper (Cu^{2+}, 1 mol/mol of enzyme). Electron paramagnetic resonance spectroscopy of the enzyme indicates that it is a type II copper-containing protein. The oxidized enzyme must be reduced by a single electron to be catalytically active. Dithiothreitol was found to be an effective reducing agent for the enzyme. Electron paramagnetic resonance data and kinetic results indicate the formation of an enzyme–thiol complex during the aerobic reduction of the enzyme by dithiothreitol. 6,7-Dimethyltetrahydropterin also reductively activates the enzyme, but only in the presence of the substrate, and is kinetically less effective than dithiothreitol. The metal center is not reoxidized as a result of normal turnover. However, the data indicate an alternative pathway exists that results in slow reoxidation of the enzyme. The 4a-hydrate of 6-methyltetrahydropterin (4a-carbinolamine) is observed during turnover of the enzyme. This intermediate is also observed during the reaction catalyzed by the iron-containing mammalian enzyme, suggesting that the mechanism of oxygen activation is similar for both enzymes.

Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) catalyzes the stoichiometric formation of tyrosine from phenylalanine and molecular oxygen. The enzyme requires a tetrahydropterin cofactor, which is oxidized by two electrons during each turnover, for activity. The mammalian enzyme contains 1 mol equiv of essential non-heme iron (Fisher et al., 1972; Gottschall et al., 1982). For catalytic activity ferric phenylalanine hydroxylase (PAH)[1] must be reduced to

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the ferrous state in an initial reduction step (Marota & Shiman, 1984; Wallick et al., 1984). There is no net reoxidation of the iron during normal turnover. While the specific role of the metal in the mechanism of the reaction is not known, the intermediacy of a peroxypterin–iron complex has been proposed (Benkovic et al., 1985a).

Non-heme iron is not only an important feature of the reaction catalyzed by the mammalian enzyme, but it is also necessary in the reaction catalyzed by *Pseudomonas* PAH (Guroff & Ito, 1965). However, PAH isolated from *Chromobacterium violaceum* was found to contain no iron and had no apparent iron requirement for activity (Nakata et al., 1979). Thus it is possible to question whether there is an absolute requirement for a metal ion in the mechanism of pterin-dependent hydroxylases. However, the apparent discrepancy in metal ion requirement could be easily resolved if the enzyme from *C. violaceum* perhaps used a different metal ion other than iron in catalysis. This paper describes our investigation regarding this possibility. The data indicate that the enzyme is a copper-containing monooxygenase. The enzyme requires an initial reduction of Cu^{2+} to Cu^{+} for activation and thereafter displays no net reoxidation during normal turnover. The kinetics of the reduction step by $DMPH_4$ and by DTT have been investigated. Some essential mechanistic features appear similar to those observed for the mammalian liver enzyme (Lazarus et al., 1981, 1982a; Wallick et al., 1984; Marota & Shiman, 1984) and support an important role of transition metals in the mechanism of pterin-dependent hydroxylases.

Experimental Procedures

Materials

6,7-Dimethyltetrahydropterin ($DMPH_4$) and 6-methyltetrahydropterin ($6MPH_4$) were prepared by the methods of Mager et al. (1967) and Storm et al. (1971), respectively. Reduced pterin was prepared by catalytic hydrogenation over Pt/C. Catalase was obtained from Boehringer-Mannheim as a crystalline suspension and was diluted to 1 mg/mL in 20 mg/mL in 20 mM Hepes, pH 7.4, before use. L-Tyrosine was purchased from Nutritional Biochemicals Corp. and was recrystallized from water. Bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), flavin adenine dinucleotide (FAD), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 2-(*N*-morpholino)ethanesulfonic acid (MES), 1,1-diphenyl-2-dipicrylhydrazyl, NADH, L-phenylalanine, and metal standards were purchased from Sigma Chemical Co. Dithiothreitol (DTT) was obtained from either Aldrich Chemical Co. or Sigma Chemical Co. 1-Nitroso-2-naphthol was obtained from Eastman Chemical Co. Doubly distilled water was used throughout these studies. All other reagents were of the highest grade commercially available.

Methods

UV and visible spectra were recorded on Cary 118, Cary 219, or Perkin-Elmer Lambda array 3840 spectrophotometers. Fluorescence was measured on a Perkin-Elmer MPF 44A instrument. Kinetic assays were performed on a Gilford 240 or 252 instrument. pH measurements were made by using a Radiometer 22 instrument equipped with a Model PHA 630 Pa scale expander and a Radiometer GK-2302C electrode. Metal determination was carried out with a Perkin-Elmer 703 atomic absorption spectrophotometer equipped with a graphite furnace.

Protein Purification. PAH from *C. violaceum* was purified by modification (Pember et al., 1986) of the procedure described by Nakata et al. (1979). DHPR was purified from pig liver as described by Craine et al. (1972). The purity of the enzymes used in these studies was 95% or better as estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis performed by the method of Laemmli (1970). The specific activity of PAH used in this work ranged from 10 to 12.9 units/mg of protein. The specific activity of DHPR was 57 units/mg of protein.

Assays. Protein was estimated by the method of Lowry et al. (1951). When samples were in sulfonic acid buffers, the Bensadoun and Weinstein (1975) modification of the Lowry procedure was used. DHPR was assayed by the method of Craine et al. (1972). Crystalline bovine serum albumin was used as a standard. PAH activity was determined at 25 °C by monitoring the change in absorbance at 275 nm (ϵ = 1.7 mM^{-1} cm^{-1}) due to tyrosine formation (Miller et al., 1975), by following the rate of NADH oxidation at 360 nm (ϵ = 4.4 mM^{-1} cm^{-1}) in a coupled system using DHPR to reduce cofactor oxidized during hydroxylation (Kaufman, 1957), or by measuring tyrosine formation from the fluorescence of the nitrosonaphthol derivative (Waalkes & Udenfriend, 1957) relative to appropriate standards. All assays were conducted by using air-saturated buffer unless otherwise indicated. Metal content of *C. violaceum* PAH was determined by atomic absorption spectrophotometry using the method of standard addition to the samples. All samples for metal determination were dialyzed overnight against 10 mM MES buffer, pH 6.5, containing 100 mM NaCl. Samples were analyzed in duplicate with 2–2.5 μg of protein. Since *C. violaceum* PAH is monomeric (Nakata et al., 1979), metal content is reported per mole of enzyme (M_r 32 000). Dialysis tubing was treated in a boiling bicarbonate–EDTA bath to remove adventitious metals before use.

Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR measurements were made on a Varian E-line spectrometer operating at the X-band. A Model E-102 microwave bridge with reference arm was used. All measurements were made at liquid nitrogen temperatures (77 K). 1,1-Diphenyl-2-dipicrylhydrazyl was used as the magnetic field standard. In some cases, repetitive scans were digitized, averaged, and stored on magnetic disk by using a Model S-100 microcomputer (Centre County Computer Consultants). Cu^{2+}–EDTA was prepared as described by Malmström and Vänngård (1960). Copper concentration was determined under nonsaturating conditions by double integration of spectra as described by Poole (1967). When the copper content of PAH was estimated by using a Cu^{2+}–EDTA standard, identical instrumental settings were used for quantitative comparison. These were as follows: frequency, 9.08 GHz; power, 20 mW; modulation, 8 G; scan time, 8 min; gain, 2×10^3; field set, 2900 G. In these experiments PAH was in 50 mM MES, pH 6.5, containing 100 mM NaCl. The theoretical variation of EPR signal intensity(s) with microwave power (P) was determined from the semiempirical equation given by Beinert and Orme-Johnson (1967):

$$\log (S/P^{1/2}) = C - 0.5b \log (1 + P/P_{1/2}) \qquad (1)$$

where C is a constant, b is an inhomogeniety parameter that

[1] Abbreviations: PAH, phenylalanine hydroxylase; pterin, generic name for 2-aminopteridin-4-one; $DMPH_4$, 6,7-dimethyltetrahydropterin; $DMPH_2$, 6,7-dimethyldihydropterin; $6MPH_4$, 6-methyltetrahydropterin; $6MPH_2$, 6-methyldihydropterin; 4a-OH-$6MPH_4$, the 4a-hydroxy adduct of $6MPH_4$; DHPR, dihydropterin reductase; EPR, electron paramagnetic resonance; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate. For additional structural details on the dihydropterin species see Benkovic et al. (1985b).

can vary from 1 (inhomogeneous broadening) to 3 (homogeneous broadening), and $P_{1/2}$ is the microwave power at half-saturation. Aerobic titrations of PAH with DTT or $DMPH_4$ were performed in standard quartz EPR tubes. After addition of reductant, samples were incubated on ice for approximately 5 min prior to refreezing. Freezing and thawing controls were done (eight cycles) to ensure that there were no changes in the spectra.

Anaerobic Procedures. Solutions were deoxygenated on an anaerobic gas train (Williams et al., 1979) utilizing argon. When anaerobic EPR experiments were performed, a quartz EPR tube fitted to a three-way stopcock was used to deoxygenate the sample as well as to make anaerobic additions under positive argon flow. Sodium dithionite was dissolved in deoxygenated 20 mM HEPES buffer, pH 8.2, and standardized by reductive titration of FAD at pH 7.0. An extinction coefficient of 11 300 M^{-1} cm^{-1} at 450 nm was used for oxidized FAD. Standardization was performed with a spectrophotometric anaerobic titration cell (Williams et al., 1979). All anaerobic transfers were made with gastight syringes under positive argon flow.

Determination of Cofactor Used to Product Formed under Conditions of Low Enzyme Turnover. The quantity of tyrosine produced relative to $DMPH_4$ used was determined under conditions where the molar ratio of $DMPH_4$ to PAH was varied from 0.24 to 40.0 in the presence of saturating L-phenylalanine (1 mM). All reactions were performed at 25 °C in air-saturated buffer (80 mM Hepes, pH 7.4) containing 15 μg of catalase in a total volume of 0.3 mL. A 3 μM PAH sample was used in each experiment. The reactions were initiated by the addition of pterin and then quenched after 15 min by the addition of nitrosonaphthol reagent for the fluorometric determination of tyrosine. The time course of tyrosine production was determined at a mole ratio of $DMPH_4$ to PAH of 2.6. The conditions were as described above, but in a total volume of 0.6 mL with 30 μg of catalase. Aliquots of the reaction mixture were removed at appropriate intervals and quenched into nitrosonaphthol reagent. Under the conditions of the experiments all $DMPH_4$ was oxidized to $DMPH_2$ during the course of the reaction. This was determined by the extent of pterin-dependent dichloroindophenol reduction ($\epsilon_{600nm} \simeq$ 16.0 mM^{-1} cm^{-1}, oxidized) when compared to dye reduction with fully reduced pterin (Lazarus et al., 1982b).

Identification of 4a-OH-6MPH$_4$ as a Reaction Intermediate. The 4a-hydrate of $6MPH_4$ was detected by rapid-scan spectrophotometry (Perkin-Elmer Lambda Array 3840). Reactions were run at 25 °C in a 1-mL reaction volume with 18.8 μM $6MPH_4$, 1 mM L-phenylalanine, 50 μg of catalase, and 0.66 μM PAH. Oxygen-saturated buffer (80 mM Tris-HCl, pH 7.6) was used in the experiments. Reagent blanks were subtracted from the experimental spectra that contained all reaction components except $6MPH_4$. Scans were taken every 20 s from 400 to 230 nm and recorded on magnetic disk. Reactions were initiated by the addition of PAH that had been preincubated with DTT (25 mM) to reductively activate the enzyme. The concentration of $6MPH_2$ in the reaction mixture was determined by the change in absorbance of the spectra at 400 nm ($6MPH_2$, ϵ = 1.8 mM^{-1} cm^{-1}) since $6MPH_4$ and 4a-OH-$6MPH_4$ have neglible absorbance at this wavelength. Under the conditions of the experiment, the concentration of $6MPH_2$ was found to be neglible. The DTT (500 μM) in the reaction mixture reduces $6MPH_2$ back to $6MPH_4$. The initial amount of $6MPH_4$ in the reaction mixture was determined by absorbance at 300 and 320 nm ($6MPH_4$, ϵ_{300nm} = 10.75 mM^{-1} cm^{-1}, ϵ_{320nm} = 5.4 mM^{-1} cm^{-1}). During the course of the reaction the concentration of $6MPH_4$ was determined by absorbance at 320 nm since 4a-OH-$6MPH_4$ has essentially no absorbance at this wavelength. The absorbance for calculated amounts of $6MPH_4$ was subtracted from the experimental spectra, yielding a difference spectrum typical of the 4a-hydrate. This was confirmed by mass balance of the total amount of pterin in the experiment with an extinction coefficient of ϵ = 16.0 mM^{-1} cm^{-1} at 245 nm for 4a-OH-$6MPH_4$ (Lazarus et al., 1982b).

Kinetics and Computer Simulation/Optimization. The pre-steady-state lag in tyrosine formation observed in these studies was related to an observed first-order rate constant describing the conversion of enzyme activity from an initial velocity to a final observed velocity. The general equation

$$P = V_f(t) - (V_f - V_i)(1 - e^{-kt})/k \quad (2)$$

was used, where V_i and V_f represent initial and final (linear) velocities, respectively, k is the observed first-order rate constant, and P is product (tyrosine) formed at time t. Initial velocity (V_i) in these studies was zero. Product vs. time data (at least 10 experimental data pairs) were computer fit to the above equation by using a multiparameter fitting program. The program uses the "simplex" algorithm for optimization. In all experiments k was optimized and V_f was taken as the final observed steady-state velocity. All experiments were performed at 25 °C.

Kinetic sequences were computer simulated. The Adams method was used to solve the system of differential equations. The program is based on a computer simulation system for enzyme kinetics by J. Moore and B. Finzel, Department of Chemistry, Eastern Michigan University, Ypsilanti, MI 48197. Both computer programs used in this work were written by Dr. David Clark of this department. The programs are written in USCD Pascal (optimization) and Turbo Pascal (simulation) and were run on a Model S-100 microcomputer (Centre County Computer Consultants).

RESULTS

Metal Content and EPR Spectroscopy of C. violaceum PAH. By the use of atomic absorption spectrophotometry, purified PAH was analyzed for several different metals, including zinc, iron, and copper. Zinc content was 0.03 mol/mol of enzyme, and iron content was also nonstoichiometric at less than 0.30 mol/mol of enzyme in several preparations. The results of the iron determination are essentially in agreement with those of Nakata et al. (1979). Furthermore, there was no correlation of iron content with specific activity of the enzyme. However, the enzyme was found to contain 1.0 mol of copper/mol of enzyme (0.99 ± 0.05, mean ± standard deviation, n = 3).

The copper content and properties of the enzyme were also examined by using EPR spectroscopy. The spectrum of the enzyme at X-band (77 K) is shown in Figure 1. It is quite typical of the cupric ion (Cu^{2+}, $S = {}^1/_2$) in an axial coordination geometry (Boas, 1985). The principal values of the spin Hamiltonian are estimated as $g_\perp$ = 2.06, $g_\parallel$ = 2.32, and $A_\parallel$ = 0.0157 cm^{-1}. On the basis of the apparent g values, the magnitude of the nuclear hyperfine interaction, and the lack of strong visible absorption, the enzyme is characteristic of many type II copper proteins. The inset to Figure 1 describes the power saturation behavior of the enzyme-bound Cu^{2+} by using eq 1 (see Methods), which relates microwave power to signal saturation and line broadening. The data are consistent with the progressive saturation of a single copper center with half-saturation ($P_{1/2}$) at 85 mW. A value of $b \cong 0.9$ indicates that the signal is inhomogeneously broadened. Since the

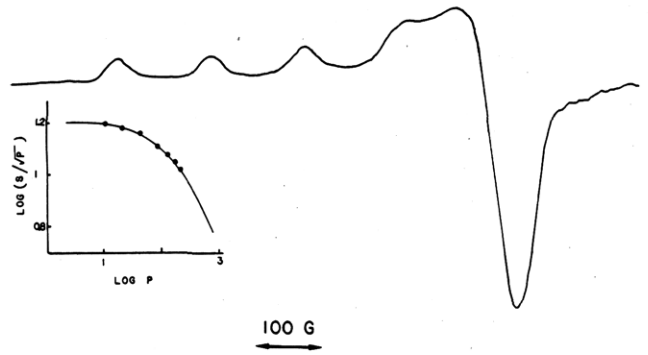

FIGURE 1: EPR spectrum of *C. violaceum* PAH. The instrument settings were as follows: frequency, 9.09 GHz, microwave power, 20 mW; modulation amplitude, 8 G; gain, 2×10^3; scan time, 4 min. The spectrum is the average of two scans and is baseline corrected. The protein concentration was 235 μM in 0.1 M HEPES, pH 7.4. The inset describes the power saturation behavior of the signal. All spectra were recorded at liquid-nitrogen temperatures (77 K).

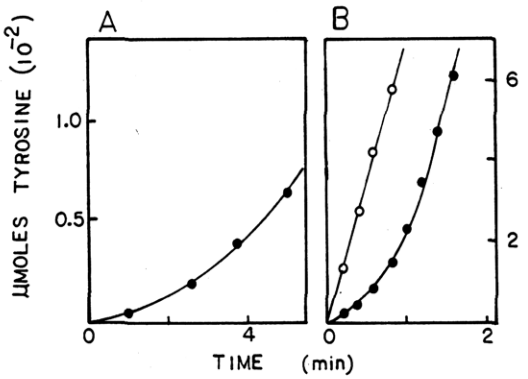

FIGURE 2: (A) Pre-steady-state lag in product formation observed during assay of PAH with DHPR used to recycle cofactor. The reaction mixture contained 5 μg of DHPR, 140 μM NADH, 50 μg of catalase, 1 mM L-phenylalanine, 165 μM $DMPH_4$, 6 μg of PAH, and 80 mM HEPES, pH 7.4, in a total volume of 1 mL. The reaction was initiated by the addition of hydroxylase. (B) Pre-steady-state lag in product accumulation during assay of the enzyme spectrophotometrically with DTT used to recycle oxidized cofactor (●). The assay medium is as described in part A except that 6 mM DTT was included and DHPR and NADH were omitted. Hydroxylase was added to initiate the reaction. Open circles denote product accumulation when PAH is preincubated in the assay medium and pterin is used to initiate the reaction.

sample was not easily saturated, the copper content of the enzyme was also estimated by comparison to a Cu^{2+}–EDTA standard. The results were in good agreement (0.95 mol of copper/mol of enzyme) with the stoichiometry determined by atomic absorption.

Hysteretic Product Accumulation. During assay of the enzyme we observed a pre-steady-state lag in the appearance of product under some conditions. This was observed in two different assay systems. As shown in Figure 2A, when the enzyme is assayed by using NADH and DHPR to recycle oxidized cofactor, slow appearance of product is observed over approximately 5 min. This behavior could not be attributed to phenylalanine activation of the enzyme, as is observed with the mammalian enzyme (Nielsen, 1969; Tourian, 1971), since preincubation with phenylalanine did not abolish the lag. The behavior was independent of the order of addition of substrate and cofactor. Similar behavior was also observed for the enzyme when assayed by using DTT to recycle the oxidized cofactor (Miller et al., 1975) as shown in Figure 2B. In this case, however, hysteretic product accumulation was dependent on the order of addition of the assay components since the behavior was observed only when the reaction was initiated

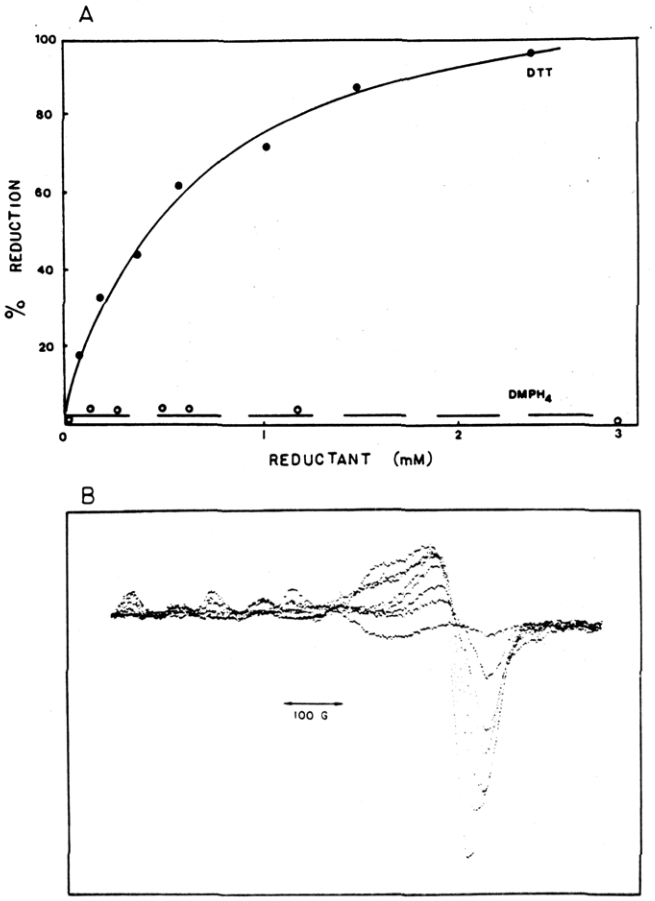

FIGURE 3: (A) Reductive titration of *C. violaceum* PAH with $DMPH_4$ and DTT by monitoring the loss of paramagnetic resonance. Experimental conditions were as described in Figure 1. The enzyme solution (50 μL) contained 1 μg of catalase. Relative signal intensities were compared by double integration of the spectra. (B) EPR spectra observed during titration of PAH with DTT. The spectra show the presence of two oxidized species of the enzyme which can be distinguished by the two sets of transitions due to copper fine structure in the $A_{\parallel}$ region. Experimental conditions are as described in part A.

by the addition of oxidized enzyme or DTT to the complete reaction mixture. Preincubation of the enzyme in an assay mixture containing DTT completely abolished the lag in product formation (Figure 2B). Two observations were made when the enzyme activity in each assay system was compared: First, when DHPR and NADH are used to recycle the cofactor the final steady-state velocity is approximately 10-fold less than the velocity observed when the enzyme is assayed by using DTT to recycle the cofactor. Second, the time to reach final linear velocity in the two systems is different. Thus, the extent and time dependency of enzyme activation are different in the two assay systems.

Reduction of the Enzyme as a Prerequisite for Catalysis. The mammalian iron-containing enzyme requires an initial conversion from ferric to ferrous form for catalytic activity (Wallick et al., 1984; Marota & Shiman, 1984). Because of this precedent, it seemed possible that the pre-steady-state lag observed in the reaction catalyzed by *C. violaceum* PAH might be related to an initial reduction of the enzyme to a cuprous form. Given this possibility, the observed inequivalence of enzyme activation in different assay systems would imply a large disparity in the ability of potential reductants to reduce the enzyme.

To explore these possibilities the oxidation state of the enzyme was examined by EPR upon aerobic titration of the enzyme with both $DMPH_4$ and DTT. Figure 3A shows the

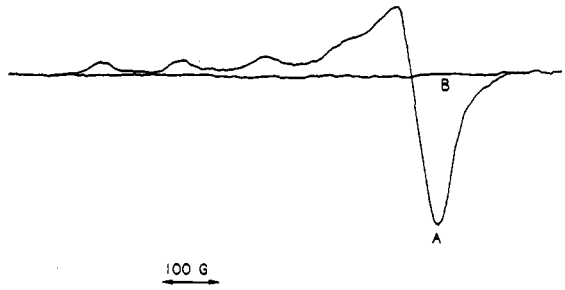

FIGURE 4: Anaerobic reduction of *C. violaceum* PAH by dithionite. The EPR spectrum of the native enzyme is depicted in spectrum A, and that of the enzyme after the addition of 0.44 equiv of dithionite is depicted in spectrum B. Instrument settings were as follows: frequency, 9.1 GHz, microwave power, 30 mW; modulation amplitude, 12.5 G; gain, 2.5×10^3; scan time, 4 min. The enzyme solution contained 210 μM PAH, 50 mM MES, pH 6.5, 50 mM NaCl, and 1 μg of catalase in a total volume of 50 μL. The spectra are the average of two scans at (77 K) and are base line subtracted.

results of such an experiment. When the enzyme was titrated with pterin, no loss of signal intensity was observed which would indicate the reduction of Cu^{2+} to diamagnetic Cu^{+}. It was possible that reduction of the copper might not be observed if there was rapid reoxidation of the metal under aerobic conditions. This situation would also predict pterin oxidase activity if the cofactor was reducing the metal. This possibility was ruled out since no pterin oxidase activity was observed upon incubation of the enzyme with the cofactor. If, however, the enzyme was titrated with DTT, progressive loss of the copper EPR signal was observed (Figure 3A), indicating the formation of cuprous enzyme. The reduction of the Cu^{2+} in the enzyme appeared to follow simple saturation behavior as shown in Figure 3A. Reduction of the enzyme did not reach a sharp endpoint, requiring the addition of excess reducing agent for complete reduction of the enzyme. Examination of the EPR spectra showed two oxidized forms of the enzyme during titration with DTT. Figure 3B shows the superimposition of the two enzyme species characterized by different sets of magnetic parameters. The first species is native oxidized enzyme, while the second species had the following estimated parameters: $g_{\perp} = 2.05$, $g_{\parallel} = 2.26$, and $A_{\parallel} = 0.0146$ cm^{-1}. The changes in g and decrease in nuclear hyperfine interaction relative to the native enzyme are suggestive of a direct coordination of thiol to the enzyme (Solomon et al., 1983; Peisach & Blumberg, 1974). Thus, during the reduction of the enzyme by thiol, at least three species are present: native enzyme, an intermediate enzyme–thiol complex, and reduced enzyme. The stoichiometry of enzyme reduction was investigated under anaerobic conditions by using standardized sodium dithionite. As shown in Figure 4 the enzyme was stoichiometrically reduced after the addition of 0.44 equiv of dithionite. Since dithionite is a 2-electron donor the data indicate that the enzyme is reduced by a single electron (0.88).

When enzyme that had been prereduced with DTT was assayed, no pre-steady-state lag[2] was observed. Also, when assays were initiated with completely prereduced enzyme, the same velocity was observed regardless of the assay system. These results indicated that the conversion of the enzyme to cuprous form is necessary for activity, and the inequivalence in enzyme activity under different assay conditions (using initially oxidized enzyme) is related to the degree of enzyme reduction.

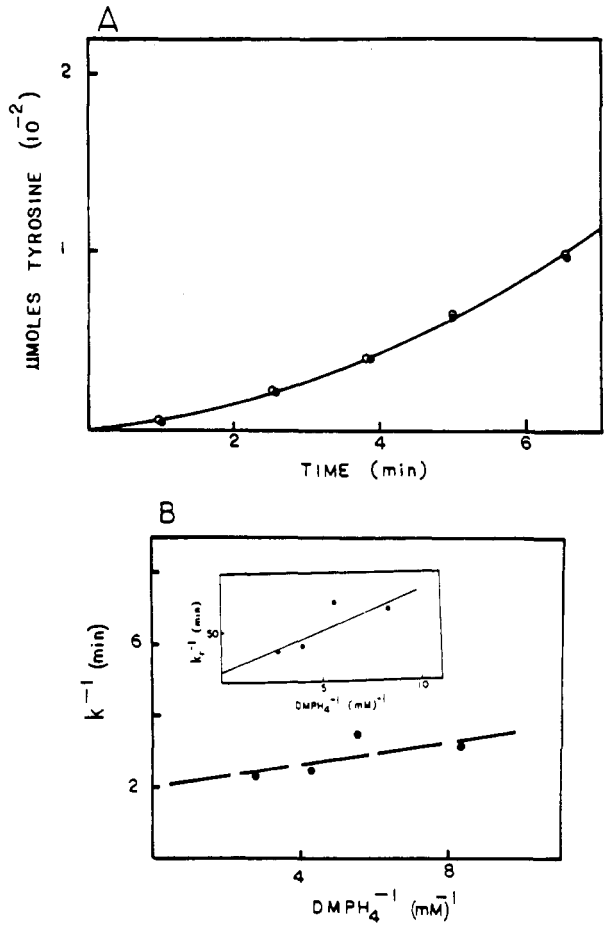

FIGURE 5: (A) Pre-steady-state lag in product accumulation with DHPR and NADH used to assay enzyme activity (O) and computer fit (●) of the data to the exponential equation described under Methods for determination of the apparent first-order rate constant (k). Experimental conditions are described in Figure 2A. (B) Plot of $1/k$ against $1/[DMPH_4]$, and a (inset) plot of $1/k_r$ against $1/[DMPH_4]$; k_r was determined from k as described under Results.

Several other reducing agents were examined for their ability to generate cuprous enzyme. These included ferrocyanide, ascorbate, and NADH; however, none were effective. Reduction of the enzyme also does not likely result in significant loss of metal from the enzyme since the activity of fully reduced enzyme is greatly inhibited in the presence of a variety of metal chelators (Pember et al., 1986).

Reduction of the Enzyme by $DMPH_4$ in the Presence of Substrate. As demonstrated above, $DMPH_4$ was unable to reduce cupric phenylalanine hydroxylase, yet tyrosine is produced in the simplest reaction mixture consisting of enzyme, phenylalanine, oxygen, and pterin cofactor (Nakata et al., 1979). Since the enzyme must be reduced for activity, the data imply that the enzyme is only reducible by pterin in the presence of substrate.

Since hysteretic product accumulation is directly related to reductive activation of the enzyme, the observed rate constants for the reduction of enzyme by pterin were determined from kinetic data by using eq 2 (see Methods). Figure 5A shows a representative computer fit of data where the rate constant (k min^{-1}) has been optimized. As can be seen the data are well represented by the single exponential process. Figure 5B shows a reciprocal plot of observed values of k at different concentrations of pterin. The observed rate of reduction appeared saturable. This suggested that an enzyme–pterin

[2] A short pre-steady-state lag of approximately 10 s can be detected when the reaction is initiated with reduced enzyme by using the enzymatic cycling assay (DHPR). This has been observed during assay of mammalian PAH and has been attributed to the initial formation of the 4a-hydrate of the pterin cofactor, which is not a substrate for DHPR (Shiman, 1985).

complex is formed prior to the reduction of the enzyme. Following the treatment of Strickland et al. (1975) the value for k observed at saturation was determined from the y intercept, yielding a value of 0.5 min^{-1}.

The final steady-state level of product formation observed when enzyme activity is monitored in absence of DTT never reaches the previously indicated steady-state level observed when the enzyme is completely reduced. This implies that the pterin-dependent reduction of the enzyme is balanced by a pathway leading to reoxidation of the reduced enzyme in the steady state. An effective steady-state equilibrium is therefore established, which can be represented under these conditions as the sum of the two first-order processes

$$E_o \xrightarrow{k_r} E_r$$

$$E_r \xrightarrow{k_o} E_o$$

where E_o is oxidized enzyme, E_r is reduced enzyme, k_r is the rate of reduction, and k_o is the rate of reoxidation. This situation is supported by the observation that the effective equilibrium can be shifted in the direction of reduced enzyme; when DTT (200 μM) is added to a reaction mixture displaying constant velocity (assay conditions as described in the caption to Figure 5A), a new, higher steady-state velocity is reached. Thus, the observed rate of reduction determined above represents the rate of approach to the steady-state level of reduced enzyme, where $k = (k_r + k_o)$.[3] The rate of enzyme reoxidation in the steady state is related to the rate of reduction by the relationship $F = k_r/(k_r + k_o)$, where k_o and k_r are as described above, and F is the fraction of reduced enzyme. The fraction of reduced enzyme was easily determined by knowing the steady-state velocity displayed by completely reduced enzyme and the steady-state velocity reached under the conditions of the experiments. Thus, at each concentration of $DMPH_4$, two equations and two unknowns allowed the determination of both k_o and k_r. When the calculated values of $1/k_r$ were plotted against the values of $1/[DMPH_4]$, extrapolation to the y intercept yielded an estimate of k_r at saturation of about 0.1 min^{-1} (inset to Figure 5B).[4] The calculated values of k_o varied only fractionally with $DMPH_4$ concentration, yielding an average value of 0.4 min^{-1}.

The ability of $DMPH_4$ to serve as a reductant for the enzyme, as well as a cofactor for phenylalanine hydroxylation, implies that if *C. violaceum* PAH is reoxidized during each turnover, then less than a stoichiometric amount of tyrosine should be produced from a given amount of pterin added in excess over the enzyme. Also, the observed rate of tyrosine formation cannot be greater than the rate of reduction of the enzyme. However, the ratio of tyrosine produced to $DMPH_4$ used is stoichiometric under such conditions (Nakata et al., 1979), and k_{cat} (463 min^{-1}) is much greater than the rate of reduction of the enzyme (k_r = 0.1 min^{-1}). From these data we conclude that *C. violaceum* PAH is not reoxidized after each turnover.

An initial reduction of the enzyme by reduced pterin in the presence of substrate does, however, predict that less than a stoichiometric amount of tyrosine should be produced from a given amount of pterin at low ratios of pterin to enzyme

[3] See the Appendix for derivation of the equation relating k to k_r and k_o and k_r and k_o to the fraction of reduced enzyme.

[4] Since the rate (k_r) is saturable, it is possible to determine a K_d for $DMPH_4$ binding to the enzyme from the kinetic data (Strickland et al., 1975). However, under the conditions of the experiment, $DMPH_4$ was nearly saturating at all concentrations used. $DMPH_4$ could not be varied over a large enough range to reliably determine K_d from the slope and intercept of the reciprocal plot.

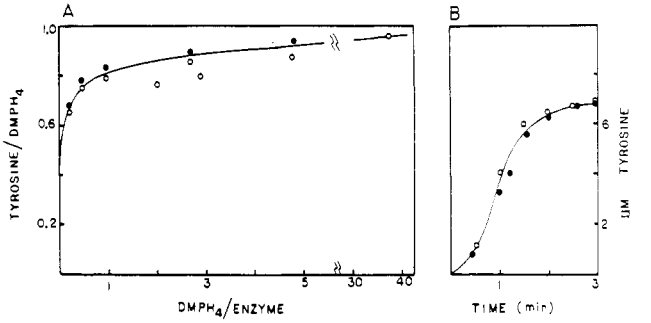

FIGURE 6: (A) Mole ratio of product formed to $DMPH_4$ used at various mole ratios of $DMPH_4$ to enzyme (○). (B) Time course of tyrosine formation at a mole ratio of $DMPH_4$ to enzyme of 2.6 (○). The experimental conditions for both A and B are described under Methods. The data in both A and B were computer simulated (●) in accord with the proposed reaction sequence (Scheme I). The values of the constants used were as follows: K_d = 4.8 μM, $K_{d'}$ = 25 μM, k_r = 0.11 min^{-1}, k_{cat} = 463 min^{-1}, and k_o = 0.4 min^{-1}.

(Wallick et al., 1984). This was observed as depicted in Figure 6A. The time course of product formation under these conditions also shows a lag in product formation (Figure 6B) indicative of an initial reduction step. These data were then simulated by using the minimal reaction sequence shown in Scheme I derived from the foregoing results, where E_o, E_r, k_r, and k_o are as described previously, and K_d and $K_{d'}$ represent apparent binding constants of $DMPH_4$ to oxidized and reduced enzyme, respectively. Under the conditions of the experiment, substrate is saturating, with E_o and E_r representing substrate complexes of oxidized and reduced enzyme, respectively. As seen in Figure 6A,B both final product to pterin ratios as well as the time course for product formation are well represented by the above reaction sequence. The values for K_d and $K_{d'}$ are the only variables optimized since k_{cat}, k_o, and k_r are known. The values for K_d (4.8 μM) and $K_{d'}$ (25 μM) used in the simulation suggest that $DMPH_4$ binds more tightly to oxidized enzyme than to reduced enzyme, although these values are subject to the presumed 1:1 stoichiometry of the reduction employed in the fitting procedure.

Scheme I

$$E_o + DMPH_4 \overset{K_d}{\rightleftharpoons} E_o{\cdot}DMPH_4 \xrightarrow{k_r} E_r + DMPH_2 + e^- + H^+$$

$$E_r + DMPH_4 \overset{K_{d'}}{\rightleftharpoons} E_r{\cdot}DMPH_4 \xrightarrow{k_{cat}} E_r + DMPH_2 + \text{tyrosine}$$

$$E_r \xrightarrow{k_o} E_o + e^- + H^+$$

Kinetics of Reductive Activation of the Enzyme by DTT. The pre-steady-state lag in product formation was also used to determine rates of reductive activation of oxidized enzyme by DTT in the presence of phenylalanine, oxygen, and cofactor. The observed first-order rate constants at different concentrations of DTT were determined by using the exponential function described previously. Under the conditions of the experiment the enzyme is reduced by both thiol and $DMPH_4$. Product vs. time data were therefore corrected for the contributions from $DMPH_4$-dependent reduction of the enzyme prior to determination of the rate constants. A representative computer fit of the data is depicted in Figure 7. Again the data are well described by a single exponential process.

At relatively low concentrations of DTT (below 500 μM) the final observed steady-state velocity did not reach that expected for fully reduced enzyme and again indicated the presence of a competitive pathway leading to reoxidation of

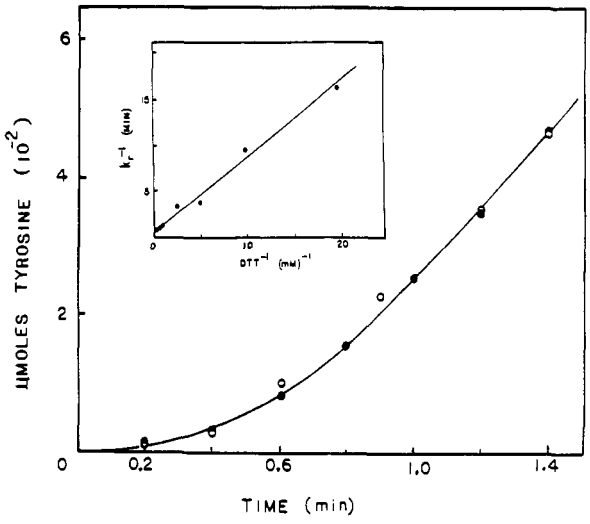

FIGURE 7: Hysteretic product accumulation monitoring tyrosine formation in the presence of 6 mM DTT (●). The data were computer fit to the exponential equation under Methods where the observed rate constant (k) has been optimized (○). The inset is a plot of $1/k_r$ against $1/[DTT]$. k_r was determined as described under Results. At 0.5 mM DTT and below enzymatic cycling (DHPR) was used to assay the enzyme. At these concentrations DTT did not interfere appreciably with enzymatic reduction of $DMPH_2$. The assay mixture included in a total volume of 1 mL 80 mM HEPES, pH 7.4, 50 μg of catalase, 1 mM L-phenylalanine, 140 μM NADH, 5 μg of DHPR, 6 μg of PAH, 180 μM $DMPH_4$, and DTT. At 1 mM DTT and higher concentrations the enzyme was assayed spectrophotometrically by measuring tyrosine formation at 275 nm (see Methods). The components of the assay solution were as described above except that DHPR and NADH were omitted.

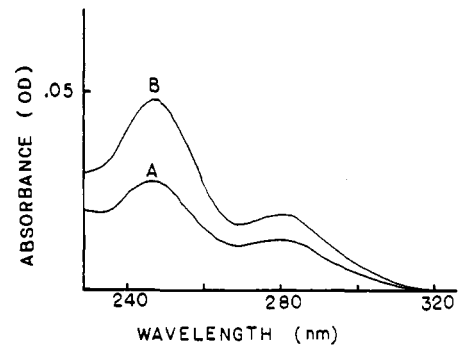

FIGURE 8: Sequential UV spectra of 4a-OH-$6MPH_4$ generated during turnover of *C. violaceum* PAH. Spectrum A was recorded 20 s after initiation of the reaction. Spectrum B was recorded when the change at 244 nm had reached a maximum (60 s). Experimental conditions are given under Methods.

the enzyme. The observed rate constants for reductive activation again represent the rate of approach to the steady-state level of reduced enzyme. From the relationships described previously, both the rate of reduction (k_r) and the rate of reoxidation (k_o) were determined for each observed value of k. Both k and k_r are saturable with respect to DTT, with an average value for $k_o = 0.4$ min^{-1} in agreement with that for $DMPH_4$. At higher levels of DTT the concentration of oxidized enzyme is negligible in accord with the EPR data presented earlier. A plot of $1/k_r$ against the values of $1/[DTT]$ is linear (Figure 7) indicating a two-step sequence (Strickland et al., 1975) as indicated in Scheme II, where E_o and E_r are as described previously and DTT_r and DTT_o represent reduced and oxidized DTT respectively. From the y intercept k_r was determined as 1.3 min^{-1}, and from the ratio of slope to intercept the value for K_d was 1.1 mM. The degree of complexation between oxidized enzyme and thiol is in good agreement with the EPR data, which showed such an intermediate directly.

Scheme II

$$E_o + DTT_r \overset{K_d}{\rightleftharpoons} E_o{\cdot}DTT_r \xrightarrow{k_r} E_r + DTT_o + e^- + H^+$$

4a-OH-6MPH$_4$ Is an Intermediate Formed during Hydroxylation. The 4a-hydrate of the pterin cofactor is an important intermediate formed concomitantly with tyrosine production (Kaufman, 1975; Lazarus et al., 1981, 1982a,b) catalyzed by the mammalian enzyme. This intermediate is characterized by an absorption maximum at 244 nm and was also observed during the reaction catalyzed by the bacterial enzyme as shown in Figure 8. As seen, there was a time-dependent increase in the concentration of the intermediate, which reached a steady-state level after about 1 min. Under the conditions of the experiment dehydration of the intermediate was not observed due to the presence of DTT (for fully reduced enzyme), which quickly reduces $6MPH_2$ back to $6MPH_4$. However, the presence of the intermediate was confirmed by mass balance of the total amount of pterin (18.8 μM) in the experiment using the known extinction coefficients for 4a-OH-$6MPH_4$ and $6MPH_4$. Under the conditions of the experiment the maximum concentration of 4a-OH-$6MPH_4$ observed was 3 μM. We were not able to observe the 4a-hydrate of the cofactor using $DMPH_4$. The inability to observe the 4a-hydrate by using $DMPH_4$ has also been reported for the mammalian enzyme (Lazarus et al., 1981).

Discussion

Copper-containing PAH from *C. violaceum* shares several important mechanistic features with the iron-containing enzyme from mammalian liver. Both enzymes require a transition metal for activity that must be reduced to yield catalytically active enzyme. Once reduced, the corresponding metal center displays no net reoxidation as part of the normal catalytic cycle. Both enzymes use tetrahydropterin in two distinct reactions: the first is the pterin-dependent reduction of the corresponding metal center by one electron, and the second is pterin-dependent hydroxylation of phenylalanine. In addition both enzymes produce the 4a-hydrate of the pterin cofactor during turnover. This suggests that ultimately the mechanism of oxygen activation is the same in both enzymes regardless of the nature of the transition metal.

In contrast to mammalian PAH the bacterial enzyme is not reduced by tetrahydropterin in the absence of substrate. Whereas mammalian PAH is reduced by tetrahydropterin ($6MPH_4$) with a second-order rate constant of $k_r = 2 \times 10^4$ M^{-1} s^{-1} (Wallick et al., 1984) in the presence of phenylalanine, *C. violaceum* PAH is reduced by $DMPH_4$ at saturation with a first-order rate constant of $k_r = 0.1$ min^{-1}. Thus, the kinetics associated with the pterin-dependent reduction of bacterial PAH are indicative of a preequilibrium binding step between pterin and enzyme. It is clear that reduction of *C. violaceum* PAH by $DMPH_4$ is rate-limiting in the minimal reaction scheme presented (Scheme I). Although *C. violaceum* PAH is not reoxidized as a result of normal turnover, a pathway exists that results in slow reoxidation of the enzyme, further limiting the observed steady-state velocity. The effective equilibrium between oxidized and reduced enzyme lies greatly in the direction of oxidized enzyme since the rate of reduction of the enzyme by $DMPH_4$ is less than the rate of reoxidation. Only a fraction of the total enzyme is ever reduced in the presence of $DMPH_4$. This results in the nearly complete coupling of cofactor used to product formed. The only exception is at the very lowest ratios of $DMPH_4$/PAH under the limiting conditions of the experiment depicted in Figure 7A,B.

The reduction of PAH by $DMPH_4$ was simulated by employing a single equivalent of $DMPH_4$. Since $DMPH_4$ can transfer two electrons, but the enzyme is reduced by one electron, an electron may be lost through reaction with oxygen or a disproportionation mechanism. Since the enzyme is monomeric (Nakata et al., 1979), this hypothesis is not unreasonable.

DTT, while not essential to the catalytic activity of the enzyme, is capable of reducing the enzyme in the absence of substrate and is kinetically a better reductant than $DMPH_4$ under the same conditions (k_r = 1.3 min^{-1}). Reductants such as ascorbate (E_m = 0.060 V) and ferrocyanide (E_m = 0.36 V) that are effective in reducing other copper proteins (Wherland & Gray, 1977; Goldberg & Pecht, 1976; Hamilton, 1981; Ljones et al., 1978) were not effective in generating cuprous PAH. Since the enzyme was reduced by DTT (E_m = −0.33 V) the data suggest that the enzyme may have a somewhat more negative midpoint potential than those typically found in copper proteins, which generally range from 0.2 to 0.8 V (Farver & Pecht, 1984; Hamilton, 1981; Walker et al., 1977; Ljones et al., 1978). As a chemical precedent for this possibility, mononuclear copper complexes with thiol-containing ligands have been prepared with reported midpoint potentials of −0.1 V (Gisselbrecht & Gross, 1982). The observation that $DMPH_4$ can reduce the copper center in the presence of substrate suggests that the midpoint potential of the copper might be altered upon phenylalanine binding.

The observed reduction rate of the enzyme by either DTT or $DMPH_4$ is slow relative to the rate of reduction of mammalian PAH by $6MPH_4$ (Wallick et al., 1984). The observed rates of reduction of some "blue" copper proteins by inorganic compounds ($FeEDTA^{2-}$, $Fe(CN_6)^{4-}$) are also very fast relative to those for *C. violaceum* PAH. For example the second-order rate constants for the reduction of azurin by $FeEDTA^{2-}$ and for plastocyanin and fungal laccase B by $Fe(CN)_6^{4-}$ are 1.3 × 10^3 M^{-1} s^{-1}, 1.9 × 10^4 M^{-1} s^{-1}, and 1.5 × 10^6 M^{-1} s^{-1}, respectively (Wherland et al., 1975; Fensom, quoted in Wherland and Gray, 1977; Andréasson et al., 1973). The cuprous–cupric self-exchange rate is 5 × 10^7 M^{-1} s^{-1} (McConnel & Weaver, 1956), and reverse charge transfer between copper and thiolate in azurin has a rate constant of 6.3 × 10^{-11} s^{-1} (Wiesenfeld et al., 1980). Since the intrinsic rate of electron transfer must be fast, a slow step(s) must dominate the reductive activation of *C. violaceum* PAH. The EPR parameters of the cupric enzyme indicate that it has predominately axial coordination. Since cuprous complexes are typically of tetrahedral or linear geometry the conversion of the cupric enzyme to the cuprous form is probably dominated by a relatively large reorganizational energy. The fast electron-transfer rates in type I copper proteins ("blue") are thought to be, in part, related to the tetrahedral geometry of the oxidized proteins (Farver & Pecht, 1984; Wiesenfeld et al., 1980), leading to minimal reorganizational energy upon conversion to the cuprous form.

DTT appears to form a coordination complex with *C. violaceum* PAH during the reduction of the enzyme. The change in the EPR parameters suggest that the thiol most likely occupies an equatorial coordination position on the cupric enzyme (Getz & Silver, 1974; Peisach & Blumberg, 1974). The kinetic data are consistent with the reduction of the enzyme by a single equivalent of DTT (Scheme II). This stoichiometry has also been observed in the reduction of cupric bleomycin by DTT (Freedman et al., 1982). Like $DMPH_4$, DTT transfers two electrons. Scheme II implies that an electron is lost during enzyme reduction through either disproportionation or perhaps reaction with oxygen as suggested previously in the case of $DMPH_4$.

From the current studies it is evident that at least two pathways exist for regulation of enzyme activity. Substrate availability regulates reductive activation of the enzyme by $DMPH_4$. A similar method of regulation is observed in flavoprotein hydroxylases. Reduction of the flavin cofactor by NADPH during turnover is promoted by the presence of substrate (White-Stevens et al., 1972; Howell et al., 1972). It is also clear that the presence of thiol can greatly effect the steady-state level of enzyme activity by shifting the effective equilibrium between oxidized and reduced enzyme.

Appendix

The observed first-order rate constant (k) describing hysteretic product accumulation is related to rates of reductive activation (k_r) and reoxidation (k_o) of *C. violaceum* PAH as follows:

The observed enzyme velocity (V_o) is related to final (V_f) and initial (V_i) linear velocities of fully reduced and oxidized enzyme, respectively

$$V_o = V_f(F_t) + V_i(1 - F_t) \quad (3)$$

where F_t is the fraction of reduced enzyme at a time t. Rearranging

$$(V_o - V_i)/(V_f - V_i) = F_t = C^R{}_t/C^T \quad (4)$$

where C^R is the concentration of reduced enzyme at a time t, and C^T is the total enzyme concentration. The following rate equation is applicable considering the reactions for reduction of oxidized enzyme (C^o) and reoxidation of reduced enzyme:

$$dC^R/dt = k_r(C^o) - k_o(C^R) \quad (5)$$

Consideration of the mass balance relationship, $C^T = C^o + C^R$, and substitution for C^o gives

$$dC^R/dt = (k_r)C^T - C^R(k_r + k_o) \quad (6)$$

Letting $\alpha = k_r + k_o$ and $\beta = k_rC^T$, we have

$$dC^R/dt = \beta - (C^R)\alpha \quad (7)$$

which upon integrating, resubstituting for β, and rearranging yields

$$C^R{}_t = (k_rC^T/\alpha)(1 - e^{-\alpha t}) \quad (8)$$

Substituting eq 8 into eq 4 gives

$$dp/dt = V_o = V_i + (V_f - V_i)(1 - e^{-\alpha t})(k_r/\alpha) \quad (9)$$

V_i in these studies is zero, which simplifies eq 9. Integrating with respect to time gives the expression for the progress of product accumulation as described under Methods.

$$P = V_f'(t) - V_f'(1 - e^{-\alpha t})/\alpha$$

In this case the observed final linear velocity is $V_f' = (F)V_f$, where $F = k_r/(k_r + k_o)$ = the fraction of reduced enzyme, and $\alpha = k = (k_r + k_o)$.

Registry No. PAH, 9029-73-6; DTT, 3483-12-3; $DMPH_4$, 611-54-1; $6MPH_4$, 942-41-6; 4a-OH-$6MPH_4$, 83387-39-7; Cu, 7440-50-8.

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