Registry No. EC 3.1.4.7, 9013-53-0; PNPdT, 2304-08-7; PNPdTp, 24418-11-9; PNPdTp*Me, 118375-50-1; L-Tyr, 60-18-4; L-Arg, 74-79-3; L-Phe, 63-91-2.

References

Alexandrescu, A. T., Mills, D. A., Ulrich, E. L., Chinami, M., & Markley, J. L. (1988) *Biochemistry 27*, 2158-2165.

Baillargeon, M. W. (1980a) Ph.D. Thesis, Purdue University, p 47.

Baillargeon, M. W., Laskowski, M., Neves, D. E., Porubcan, M. A., Santi, R. E., & Markley, J. L. (1980b) *Biochemistry 19*, 5703-5710.

Bartlett, P. A., & Marlowe, C. K. (1987) *Science 235*, 569-571.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273-387.

Cleland, W. W. (1979a) *Anal. Biochem. 99*, 142-145.

Cleland, W. W. (1979b) *Methods Enzymol. 63*, 103-138.

Cotton, F. A., laCour, T., & Hazen, E. E. (1977) *Biochim. Biophys. Acta 481*, 1.

Cotton, F. A., Hazen, E. A., & Legg, M. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2551-2555.

Cuatrecasas, P., Wilchek, M., & Anfinsen, C. B. (1969) *Biochemistry 8*, 2277.

Dunn, B. M., DiBello, C., & Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 4769-4774.

Edsall, J. T., & Wyman, J. (1958) *Biophys. Chem. 1*, 463-471.

Glinski, R. P. L., Ash, A. B., Stevens, C. L., Sporn, M. B., & Lazarus, H. M. (1971) *J. Org. Chem. 36*, 245-249.

Grissom, C. B., Alexandrescu, A. T., Ulrich, E. L., Mills, D. A., & Markley, J. L. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1915.

Hazen, E. E., & Cotton, F. A. (1978) *Mol. Cell. Biochem. 22*, 67-77.

Hibler, D. W., Stolowich, N. J., Reynolds, M. A., Gerlt, J. A., Wilde, J. A., & Bolton, P. H. (1987) *Biochemistry 26*, 6278-6286.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, pp 250-253, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70-80.

Rammler, D. H., Yengoyan, L., Paul, A. V., & Bax, P. C. (1967) *Biochemistry 6*, 1828-1837.

Segel, I. H. (1975) *Enzyme Kinetics*, pp 915-918, Wiley, New York.

Serpersu, E. H., Shortle, D., & Mildvan, A. S. (1986) *Biochemistry, 25*, 68-77.

Serpersu, E. H., Shortle, D., & Mildvan, A. S. (1987) *Biochemistry 26*, 1289-1300.

Shortle, D. (1983) *Gene 22*, 181-189.

Shortle, D. (1986) *J. Cell. Biochem. 30*, 281-289.

Tucker, P. W., Hazen, E. E., & Cotton, F. A. (1979a) *Mol. Cell. Biochem. 23*, 3-16.

Tucker, P. W., Hazen, E. E., & Cotton, F. A. (1979b) *Mol. Cell. Biochem. 23*, 67-86.

Tucker, P. W., Hazen, E. E., & Cotton, F. A. (1979c) *Mol. Cell. Biochem. 23*, 131-141.

Uhlmann, E., & Smith, J. A. (1987) *Nucleosides Nucleotides 6*, 331-334.

Mechanistic Studies on Phenylalanine Hydroxylase from *Chromobacterium violaceum*. Evidence for the Formation of an Enzyme–Oxygen Complex†

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ABSTRACT: Steady-state kinetic analysis of pterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* indicated that the enzyme follows a partially ordered reaction mechanism. The data suggested that oxygen is the first substrate to bind to the enzyme. This result was further supported by rapid-quench experiments in which the enzyme–oxygen complex was trapped to yield product. Additional support for the presence of an enzyme–oxygen complex was derived from magnetic susceptibility measurements of molecular oxygen in the presence and absence of cuprous phenylalanine hydroxylase. The magnetic susceptibility of dissolved oxygen decreased in the presence of the enzyme, supporting a direct oxygen–metal interaction.

Phenylalanine hydroxylase (EC 1.14.16.1) catalyzes the reaction

$$H_4\text{-pterin} + \text{phenylalanine} + O_2 \rightarrow \text{tyrosine} + H_2O + H_2\text{-pterin}$$

The process requires a tetrahydropterin cofactor that is oxidized by two electrons during substrate hydroxylation. Phenylalanine hydroxylase (PAH)[1] from rat liver and from *Chromobacterium violaceum* are both metalloenzymes. Mammalian PAH contains tightly bound non-heme iron

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[1] Abbreviations: PAH, phenylalanine hydroxylase; $DMPH_4$, 6,7-dimethyltetrahydropterin; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Phe, L-phenylalanine; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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(Fe^{3+}) (Gottschall et al., 1982; Fisher et al., 1972), whereas *C. violaceum* PAH contains copper (Cu^{2+}) (Pember et al., 1986). Both enzymes require an initial single-electron reduction of their respective metal centers for catalytic activity (Marota & Shiman, 1984; Wallick et al., 1984; Pember et al., 1986). A 4a-OH derivative of tetrahydropterin is produced as an intermediate in the reaction (Kaufman 1975; Lazarus et al., 1981). Because flavin-dependent hydroxylases are thought to generate an analogous 4a-hydrated species (4a-OH-flavin), similar 4a-hydroperoxy intermediates have been suggested as possible hydroxylating species for both classes of enzymes (Ballou, 1982; Hamilton, 1974; Bruice, 1982; Dmitrienko et al., 1977). Isotopic labeling experiments are consistent with the formation of a 4a-peroxy pterin or a 4a-peroxy pterin–metal species along the reaction pathway (Ayling & Bailey, 1982; Dix et al., 1985). However, clear differences must exist in the respective mechanisms for activation of dioxygen by virtue of the metal ion dependence of pterin-dependent hydroxylation and the lack of metal ion participation in flavin-dependent hydroxylations.

While the general kinetic mechanism for flavin-requiring hydroxylases has been studied in detail by both steady-state and rapid kinetic methods (Massey & Hemmerich, 1975; Entsch et al., 1976; Detmar & Massey, 1984; Ballou, 1982), corresponding studies on pterin-dependent hydroxylases have been more limited. Initial characterization of the rat liver PAH kinetic mechanism using steady-state methods suggested a sequential mechanism for the addition of substrates rather than a ping-pong type reaction (Kaufman & Fisher, 1974). Attempts to gain additional information about the kinetic mechanism using steady-state or pre-steady-state approaches have been complicated by the necessity for phenylalanine activation of rat liver PAH for maximum enzyme activity (Neilsen, 1969; Shiman & Gray, 1980). Because substrate activation is slow (Shiman & Gray, 1980), interpretation of initial rate data in the absence of enzyme preactivation is difficult. In contrast to the mammalian enzyme *C. violaceum*, PAH does not appear to require substrate activation for maximum activity (Pember et al., 1986), simplifying analysis of the mechanism by either steady-state or rapid kinetic methods. For this reason, we have used *C. violaceum* PAH in the current study to further probe the kinetic mechanism of the enzyme. Steady-state and rapid-quench experiments indicate that *C. violaceum* follows a partially ordered mechanism. The data suggest that molecular oxygen is the first substrate to bind to the enzyme, a result supported by magnetic susceptibility experiments that provide evidence for the coordination of molecular oxygen by the cuprous ion in *C. violaceum* PAH.

Experimental Procedures

Materials

6,7-Dimethyltetrahydropterin ($DMPH_4$) was prepared by the method of Mager et al. (1967). Catalytic hydrogenation over Pt/C was used to reduce the 6,7-dimethylpterin. Lysozyme, bovine serum albumin, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), and L-phenylalanine were purchased from Sigma Chemical Co. L-Tyrosine was obtained from Nutritional Biochemicals Corp. and was recrystallized from water before use. Catalase was purchased from Boehringer-Mannheim and diluted to 1 mg/mL in 20 mM Hepes, pH 7.4, prior to use. Dithiothreitol (DTT) and 1-nitroso-2-naphthol were obtained from Aldrich Chemical Co. and Eastman Chemical Co., respectively. Oxygen–nitrogen mixtures were obtained from Curtis-Matheson.

Methods

UV and visible spectra were recorded on a Cary 219 spectrophotometer. Fluorescence was measured by using either Perkin-Elmer MPF-66 or MPF-44A spectrofluorometers. Oxygen uptake was measured by using a Yellow Springs oxygen monitor (Model 53) and reaction vessel equipped with a scale expander. pH was measured by using a Radiometer 22 instrument equipped with a Model PHA 630 Pa scale expander and a Radiometer GK-2302C electrode.

Protein Purification. *C. violaceum* PAH was purified by modification (Pember et al., 1987a) of the procedure described by Nakata et al. (1979). The purity of the enzyme was greater than 95% as estimated by SDS–polyacrylamide gel electrophoresis.

Assays. The $DMPH_4$ concentration was determined from UV absorption in 0.1 N HCl ($\epsilon_{262} = 16.0\ mM^{-1}\ cm^{-1}$). Protein concentrations were estimated by the Lowry procedure using bovine serum albumin as a standard. When samples were prepared in sulfonic acid buffers, the Bensadoun and Weinstein (1975) modification of the Lowry method was used. PAH activity was measured by using substrate-dependent oxygen consumption monitoring the change in absorbance at 275 nm ($\epsilon = 1.7\ mM^{-1}\ cm^{-1}$) due to tyrosine formation (Miller et al., 1975), or by measuring the fluorescence of the nitrosonaphthol derivative of tyrosine (Waalkes & Undenfriend, 1957).

Steady-State Kinetics. Initial rate data were measured by monitoring oxygen consumption and fit to a hyperbola by using the computer program of Cleland (1979). All reactions were performed in 80 mM Hepes, pH 7.4, containing 50 μg/mL catalase, 6 mM DTT, 12 μg of enzyme, and varying concentrations of cofactor and substrates in a total volume of 3 mL at 25 °C. Oxygen concentration was varied in the reaction by equilibrating the assay medium under atmospheres of defined oxygen composition using air, pure oxygen, or oxygen–nitrogen mixtures. Reactions were initiated by the addition of either phenylalanine or $DMPH_4$. All initial rates were corrected for background oxidation rates of $DMPH_4$ and DTT. Kinetic constants for each of the substrates were determined by computer fit of the data to the overall steady-state equation as defined under Results using the M lab Modeling program (Knott, 1979).

Rapid-Quench Experiments. A quench-flow apparatus constructed in this laboratory was used to obtain rapid-quench time points between 1 and 30 s (Johnson, 1987). In a typical experiment enzyme solutions contained 135 μM PAH (active sites), 110 μg/mL catalase, 26 mM DTT, and 1.2 mM O_2 in 80 mM Hepes, pH 7.0. Control enzyme solutions were identical except they contained 60 μM O_2. Oxygen concentration was varied by saturating solutions with 100% oxygen gas (1.2 mM) or a 5% O_2–N_2 mixture (60 μM). DTT was added just prior to loading the quench-flow apparatus to minimize oxidation of the thiol. The buffer in the drive syringe contained 50 μg/mL catalase and no enzyme. The drive syringe with anaerobic substrate solutions contained 277 μM $DMPH_4$, 1 mM L-phenylalanine, 50 μg/mL catalase, and 6 mM DTT in 80 mM Hepes, pH 7.0. Solutions were deoxygenated on an anaerobic gas train (Williams et al., 1979). Control substrate solutions were as above but contained, in addition, 60 μM O_2. All reactions were quenched into 2 N HCl. Product was measured fluorometrically against tyrosine standards.

The design of the quench experiments required the enzyme samples to be diluted 20-fold into the anaerobic substrate solution; thus, the volume of the drive syringe containing

substrates was 20 times larger than that of the drive syringe governing the flow of the enzyme sample. This was considered feasible because of the comparatively long reaction times (≥1 s). Drive syringes were gastight. All transfers into the quench-flow apparatus were made with gastight Hamilton syringes through Hamilton luer-lock valves. Teflon tubing was used in the apparatus to minimize any gas exchange during the duration of the experiments.

Magnetic Susceptibility Experiments. Magnetic susceptibility measurements of O_2 in the presence and absence of PAH were made by the Evans method (Evans, 1959; Phillips & Poe, 1972) using 5-mm coaxial NMR tubes. Samples were analyzed by use of a Bruker 360-MHz spectrometer. Samples (outer tube) were in D_2O and contained 600 μM O_2, 2 mM DSS, 50 μg/mL catalase, 4 mM DTT, and 88 mM Hepes, pD 7.0. Reference solutions (inner tube) were identical except that they were deoxygenated on an anaerobic train (Williams et al., 1979) using N_2. The inner tube was septum-sealed and then degassed by using a needle. Samples were oxygenated by using a stream of 50% O_2–N_2 gas and then sealed by the introduction of the inner tube. Enzyme samples were prepared (by use of fully active enzyme, 13 μg/mL) identically except they were first lyophilized and then resuspended in the D_2O solution. Splittings were determined from the analysis of the methyl protons of DSS. Because of slow depletion of O_2 from the samples due to DTT oxidation, splittings were measured as a function of time and extrapolated to zero. Experiments were initiated by adding DTT into the outer tube as the final component just prior to introduction of the reference tube.

Magnetic susceptibilities were calculated from (Phillips & Poe, 1972)

$$\chi = -\frac{3\Delta f}{4\pi f m} + \chi_o + \frac{\chi_o(d_o - d_s)}{m}$$

where χ is the mass magnetic susceptibility of the dissolved solute, f is the resonance frequency (360 MHz), Δf is the change in frequency of DSS due to the presence of the solute, m is the mass concentration of the solute, χ_o is the mass magnetic susceptibility of the solvent, and d_o and d_s are the densities of the solvent and solution, respectively. The last term was ignored in these calculations because its value is negligible under the experimental conditions. Effective magnetic moments (Phillips & Poe, 1972; Van Vleck, 1932) were calculated by using

$$\mu^2_{eff} = (3kT/N\beta^2)\chi_m$$

where k, N, β, and χ_m are Boltzman's constant, Avogadro's number, the Bohr magneton, and the molar magnetic susceptibility, respectively.

Results

Steady-State Kinetics. PAH is a terreactant enzyme requiring oxygen, phenylalanine, and the pterin cofactor for catalysis. The most general form of the velocity equation for a terreactant enzyme takes the form

$$v = VABC/(\text{constant} + (\text{coef } A)A + (\text{coef } B)B + (\text{coef } C)C + K_aBC + K_bAC + K_cAB + ABC) \quad (1)$$

The Michaelis constants for each substrate are represented by K_a, K_b, and K_c. The exact definitions of the constant and coefficient values as well as the presence of individual terms in the denominator are mechanism dependent. Our first efforts were directed at determining which terms in the general rate equation are necessary to adequately describe the reaction catalyzed by PAH. In doing so, the number of mechanistic possibilities describing the kinetic scheme of the reaction would be reduced.

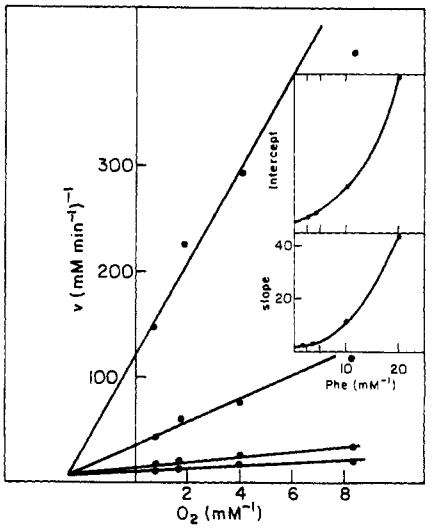


FIGURE 1: Reciprocal plots of initial steady-state velocity versus oxygen concentration for *C. violaceum* PAH. Oxygen concentrations were 1.2 mM, 584 μM, 254 μM, and 120 μM. Phenylalanine and $DMPH_4$ were held at fixed concentrations at constant ratio; concentrations were 0.5 mM Phe, 0.2 mM $DMPH_4$; 0.25 mM Phe, 0.1 mM $DMPH_4$; 0.1 mM Phe, 0.04 mM $DMPH_4$; and 0.05 mM Phe, 0.02 mM $DMPH_4$. The inset shows the slope and intercept of each line versus the corresponding concentration of Phe. See Methods for further details.

One of the most efficient protocols for analyzing terreactant systems involves holding two substrates at a constant ratio at different fixed concentrations while varying the concentration of the third substrate (Rudolf & Fromm, 1979). The initial reciprocal plots using this methodology can be diagnostic for a sequential or ping-pong type mechanism. The K_m values of the substrates then can be estimated directly from replots of slope and intercept values. In addition, the secondary plots can be used to identify specific reaction mechanisms on the basis of the linearity or nonlinearity that results from holding the two fixed substrates at a constant ratio.

The results of this type of analysis using *C. violaceum* PAH are shown in Figures 1–3. The primary reciprocal plots, in which each of the three substrates was varied at fixed concentrations of the other two, all show intersecting lines. This is indicative of a sequential mechanism. The secondary plots of both slope and intercept data for the case in which oxygen was the varied substrate (inset, Figure 1) are clearly nonlinear. However, the replots of slope and intercept data where phenylalanine and $DMPH_4$ were the varied substrates (insets, Figures 2 and 3, respectively) show slope plots that appear slightly parabolic, whereas the intercept plots are linear.

On the basis of the results from the secondary plots, many sequential mechanisms can be eliminated. For example, a completely random mechanism is eliminated because the secondary plots would be nonlinear for both slope and intercept for each substrate (Rudolph & Fromm, 1979). This analysis, of course, depends entirely upon the ability to distinguish nonlinear from linear plots, which in some cases might be ambiguous. Additionally, if a particular substrate adds more than once to the enzyme, the interpretation is problematic (Cleland, 1970). Nevertheless, the results for PAH based on the replot analysis are entirely consistent with a mechanism in which the substrate (A) adds to the enzyme followed by the random addition of the second (B) and third (C) substrates [i.e., random BC (steady-state A) ter]. The rate equation for this mechanism is then

$$v = VABC/\text{constant} + (\text{coef } A)A + K_aBC + K_bAC + K_cAB + ABC \quad (2)$$

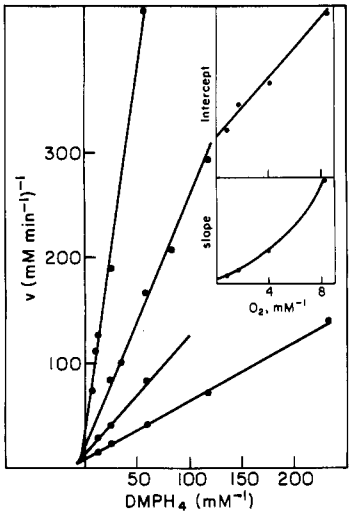


FIGURE 2: Reciprocal plots of velocity versus $DMPH_4$ concentration for PAH. $DMPH_4$ concentrations were 86.3, 43.1, 17.3, 8.70, and 4.35 μM. $DMPH_4$ concentrations were 172, 129, 86, 43.1, and 17.3 μM when oxygen concentration was 120 μM. Oxygen and Phe were at constant ratio and fixed at 120 μM O_2, 48 μM Phe; 254 μM O_2, 100 μM Phe; 580 μM O_2, 232 μM Phe; and 1.2 mM O_2, 480 μM Phe. The inset gives the slope and intercept of each line versus O_2 concentration. Further details are given under Methods.

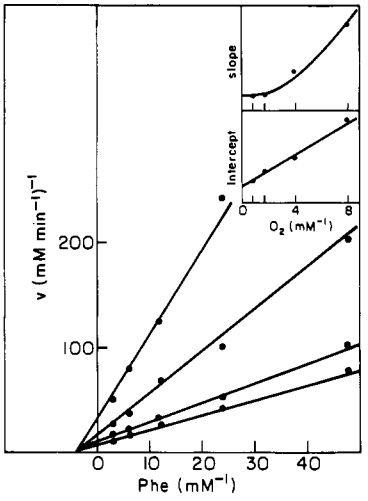


FIGURE 3: Reciprocal plots of steady-state velocity versus phenylalanine concentration for *C. violaceum* PAH. Phenylalanine concentrations used were 333, 166, 83, 42, and 21 μM. A constant ratio of oxygen and $DMPH_4$ was used at fixed concentrations; they were 120 μM O_2, 48 μM $DMPH_4$; 584 μM O_2, 234 μM $DMPH_4$; 1.21 mM O_2, 484 μM $DMPH_4$; and 254 μM O_2, 100 μM $DMPH_4$. The inset is a replot of slope and intercept values for each line at the corresponding O_2 concentration. Additional information is given under Methods.

The absence of the B and C terms give the linear intercept replots of the primary data for phenylalanine and pterin. The presence of the A term gives rise to nonlinear slope and intercept replots for oxygen and clearly implicates oxygen as the first substrate to bind to the enzyme. The values for the Michaelis constants and for V_{max} were estimated by multiparameter computer fit of the data to eq 2. The K_m (± standard error) values for oxygen, phenylalanine, and pterin are 210 ± 36, 176 ± 25, and 38 ± 10 μM, respectively; V_{max} is 34.4 ± 2.5 μmol min^{-1} mg^{-1}. The steady-state parameters were also estimated directly from the secondary plots of the initial rate data (slopes and intercepts) and were in good agreement with values derived from the computer fit.

The kinetic mechanism for *C. violaceum* appears partially ordered (obligatory addition of oxygen as the first substrate) on the basis of the experiments above. This mechanism in-

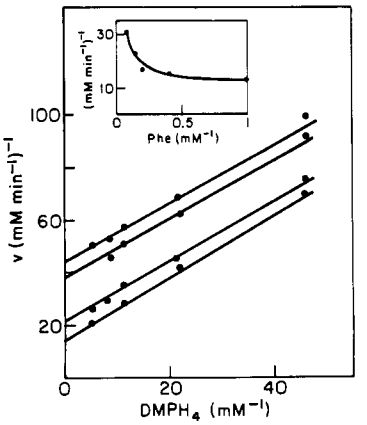


FIGURE 4: Reciprocal plots of initial velocity versus $DMPH_4$ for *C. violaceum* PAH. Phenylalanine concentration was saturating at 10 mM. Oxygen concentration was fixed at 120 μM, 254 μM, 584 μM, and 1.2 mM. The values (± standard error) for the slopes of the lines at 120 μM, 254 μM, 584 μM, and 1.2 mM O_2 are 1.1 ± 0.01, 1.1 ± 0.084, 1.11 ± 0.11, and 1.21 ± 0.02, respectively. Further details are given under Methods. The inset is a reciprocal plot of enzyme velocity versus phenylalanine concentration depicting substrate inhibition. Phenylalanine concentrations used were 1, 2, 4, 6, and 10 mM. Assays were in air-saturated 80 mM Hepes buffer, pH 7.4, containing 50 μg of catalase, 6 mM DTT, 5 μg of *C. violaceum* PAH, and 180 μM $DMPH_4$.

dicates that if phenylalanine is raised to saturating concentration (approximately 100 K_m), then the reversible connection between oxygen and the pterin cofactor in the reaction should be broken. This situation predicts that at different fixed levels of oxygen a series of parallel reciprocal plots should result when pterin concentration is varied (Frieden, 1959; Viola & Cleland, 1982; Cleland, 1970; Rudolf & Fromm, 1979). This was observed as shown in Figure 4 when phenylalanine was raised to about 60K_m. Additionally, as the concentration of phenylalanine was raised to saturating levels, substrate inhibition was observed as depicted in the inset to Figure 4. This suggests that although phenylalanine and pterin may add to the enzyme randomly, a preferred order of addition may exist in which pterin is favored over phenylalanine. Consequently, a totally ordered mechanism cannot be unequivocally excluded. Nevertheless, the results derived from saturation of the enzyme with phenylalanine complement the initial steady-state analysis described above and are supportive of a partially ordered reaction sequence.

Trapping and Enzyme–Oxygen Complex by Rapid Quench. The steady-state data indicated that oxygen is the first substrate to bind to the enzyme. This interaction was further probed by rapid-quench kinetics. The presence of a PAH–substrate complex (O_2) was probed by measuring the difference in product formation during pre-steady-state under two sets of initial conditions. Under the first set of experimental conditions a putative PAH–O_2 complex was created by equilibrating the reduced enzyme with a high concentration of oxygen (1.2 mM). The oxygen was sufficiently high (6K_m) to yield enzyme predominantly in the form of an enzyme–O_2 complex.[2] The enzyme was then rapidly diluted into an anaerobic solution containing a large amount of $DMPH_4$ and phenylalanine (the trap solution). The dilution was great enough (20-fold) to reduce the O_2 concentration to below the K_m. Thus, the putative enzyme–O_2 complex could partition either to product or to free O_2 and enzyme. If the enzyme–O_2

[2] Although the K_d for oxygen is unknown, it is reasonable to assume that the enzyme would be present primarily as a E·O_2 complex since K_m is generally > K_d.

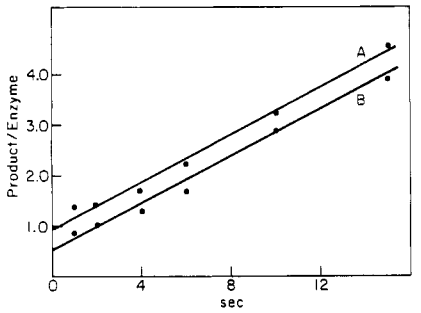


FIGURE 5: Quench-flow experiments depicting the ratio of product formed to enzyme used (P/E) versus time after preequilibrating PAH with O_2 above and below the K_m value for O_2. (A) Cuprous PAH was equilibrated at 1.2 mM O_2, rapidly diluted (20-fold) into an anaerobic trap solution containing excess $DMPH_4$ and phenylalanine, and then quenched at various intervals. (B) Cuprous PAH was equilibrated at 60 μM O_2, rapidly diluted (20-fold) into a trap solution, and quenched at intervals as above. The trap solution contained in addition 60 μM O_2. Oxygen concentration throughout the experiment was constant at 60 μM. Data were analyzed by linear regression using least squares. Further details are given under Results and Methods.

complex is trapped by cofactor and phenylalanine, then an initial burst of product would be observed relative to subsequent steady-state turnover in which the O_2 concentration is limiting ($[O_2] < K_m$). Under a second set of control conditions the enzyme was preequilibrated at a low oxygen concentration (60 μM). The enzyme was then rapidly diluted into a solution containing saturating cofactor and substrate and 60 μM O_2. Under these conditions oxygen was constant and also limiting. Any initial product burst relative to subsequent steady-state catalysis would reflect the position of the equilibrium, $E + O_2 \rightleftharpoons EO_2$ toward EO_2 upon mixing.[3] In both experiments the reactions were quenched at various intervals and the product was measured fluorometrically. The rate of product formation at the time of mixing enzyme predominantly in the form of an $E{\cdot}O_2$ complex would be near V_{max} at the concentrations of pterin and phenylalanine used in the trap solution such that the time for a single turnover would be approximately 53 ms. Because of the noise in the data and the relatively long quench times used ($\geq$1 s), the kinetics of a burst could not be observed directly. The amount of product trapped was obtained by extrapolating product formation in the steady state to zero time.

The results of one such experiment done in triplicate are depicted in Figure 5. A clear difference was observed in the amount of product formed under the two sets of experimental conditions described above. The maximum amount of product trapped (P/E, product formed/enzyme active sites) at the time of mixing was 0.88 $\pm$ 0.08 ($\pm$ standard error). The amount of product trapped (P/E) under control conditions was 0.53 $\pm$ 0.1 ($\pm$ standard error). These results provided additional support for the formation of an initial $E{\cdot}O_2$ complex. It should be noted that the steady-state turnover in both expriments depicted in Figure 5 is identical. Since the final oxygen concentration should be the same in both experiments, the steady-state velocity serves as an internal control on our manipulation of oxygen in the system.

Magnetic Susceptibility Measurements. Magnetic susceptibility was used by Pauling (1936) to study the coordination chemistry of hemoglobin with various ligands. Because

[3] Although the concentration of O_2 (60 μM) used is well below the K_m for O_2, it may not necessarily be below the K_D. Significant amounts of an $E{\cdot}O_2$ complex may therefore be present. The assumption is also made that $E{\cdot}O_2$ is trapped immediately so that there is no change in the equilibrium position upon mixing.

Table I: Magnetic Susceptibility of Dioxygen in the Presence and Absence of *C. violaceum* PAH or Lysozyme

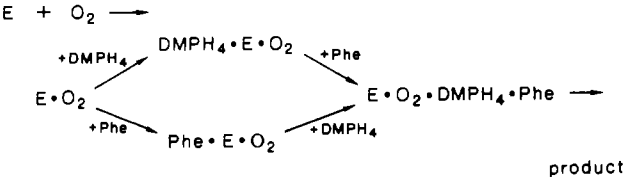
	χ ($\times 10^{-6}$)	χ_m ($\times 10^{-6}$)	μ_{eff}
O_2[a]	107.9	3452.8	2.85
O_2 + PAH (180 μM)	77.9	2492.8	2.42
O_2 + PAH (108 μM)	88.1	2819.2	2.58
O_2 + lysozyme (128 μM)	106.7	3414.4	2.83

[a] Representative values for dioxygen are in good agreement with the molar susceptibility (χ_m; 3449 $\times$ 10^{-6}) given in the *CRC Handbook of Chemistry and Physics* (1975). Data were collected on a Bruker 360-MHz NMR spectrometer using a 90° single pulse sequence. The radio frequency pulse width was 9.0 μs, the acquisition delay 4 s, the acquisition time 2.74 s, the sweep width 3000 Hz, and 48 transients were collected. Further experimental details are given under Methods.

our kinetic analysis indicated the formation of an enzyme–O_2 intermediate, we used magnetic susceptibility to investigate the nature of the interaction. Direct O_2 coordination to cuprous PAH (diamagnetic) might be expected to alter the magnetic susceptibility of paramagnetic oxygen as a result of direct electronic interaction with the metal ion (Evans, 1959). Magnetic susceptibility measurements were made on O_2 in the presence and absence of *C. violaceum* PAH. Lysozyme was used as a protein control. The results of experiments done in duplicate are given in Table I. The magnetic susceptibility of dissolved oxygen decreased in the presence of reduced PAH (Cu^+, diamagnetic) and was dependent on the concentration of enzyme. In contrast, lysozyme had no effect on the magnetic susceptibility value for oxygen. The decrease in susceptibility of oxygen is directly related to a decrease in the effective magnetic moment (μ_{eff}), indicating that the spin quantum number (S) for oxygen decreases in the presence of the enzyme. These results support an enzyme-bound state for dioxygen in which a direct O_2–copper interaction alters the susceptibility of the bound O_2.

DISCUSSION

On the basis of these studies a minimum reaction sequence for *C. violaceum* PAH includes the addition of oxygen to the enzyme as the first substrate followed by the random addition of phenylalanine and cofactor. The quaternary complex then leads to products:



Although a random sequence of addition of pterin and phenylalanine is suggested by the initial velocity experiments, it is also possible that there is a predominant order of addition where $DMPH_4$ is favored kinetically over phenylalanine as the second substrate in the reaction scheme. This is supported by the observation of substrate inhibition at high phenylalanine concentrations.

The rapid-quench experiments indicate that there is probably little desorption of O_2 from the quaternary complex by the ability to trap an $E{\cdot}O_2$ complex almost entirely to yield tyrosine (P/E = 0.88 $\pm$ 0.08) prior to steady-state turnover. Although in the current experiments the rapid-quench methods have served to identify an $E{\cdot}O_2$ complex, the method could also be used to estimate the rate of dissociation for an $E{\cdot}O_2$ complex under the appropriate conditions (Rose, 1980). Under the conditions in which PAH was preequilibrated at a low O_2 concentration (60 μM), a significant product burst was also observed (P/E = 0.53 $\pm$ 0.1). From this these data a K_D value of 50 μM can be estimated for the binding of O_2, a value that

is lower than the K_M observed for O_2, which is 210 μM. One possibility that would contribute to this difference would be the formation of dead-end complexes between cofactor or phenylalanine and PAH prior to O_2 binding to the enzyme. This idea is supported by the observation that in experiments in which cofactor or Phe is included in the preincubation solution the PAH + O_2 (60 μM) experiments yield a much smaller product burst, with P/E ranging from 0.05 to 0.21. These results suggest an obligatory formation of a binary enzyme E·O_2 complex that is effectively diluted by competing cofactor or phenylalanine.

Other copper-containing proteins such as hemocyanin and tyrosinase are thought to bind molecular O_2 through dicopper–oxygen coordination chemistry forming a μ-1,2-peroxo bridged Cu^{II} species (Solomon, 1981; Freedman et al., 1976; Eickman et al., 1978). This view is supported by various cuprous model systems reversibly binding O_2 where 1 mol of O_2 is bound for 2 mol of Cu (Karlin et al., 1987; Simmons & Wilson, 1978). The coordination of O_2 by an enzyme containing a single copper center is unusual, but is supported by the recent synthesis of a stable, Cu^{II}–superoxide complex (Thompson, 1984) reversibly binding dioxygen. Although it could be argued that cuprous PAH perhaps dimerizes, yielding a dicopper–oxygen complex, this would predict a maximum ratio of product formed to enzyme used in our stop-quench experiments of 0.5. A ratio near 1 was observed (0.88 ± 0.08), clearly supporting a Cu–dioxygen binding stoichiometry of 1:1. The decrease in magnetic susceptibility of dissolved O_2 in the presence of the enzyme is also consistent with a direct metal–dioxygen interaction.

The current studies suggest that at least one function of the metal ion in the mechanism of *C. violaceum* PAH is to coordinate molecular oxygen. The magnetic susceptibility measurements indicate that the spin state of oxygen is changed in the presence of the enzyme. Whether the bound state of the oxygen is fully diamagnetic or retains some paramagnetic character remains to be determined. On the basis of model chemistry (Thompson, 1984) a diamagnetic, spin-paired Cu^{II}–superoxide complex seems reasonable. In this configuration the conversion of normally triplet oxygen to a nonradical state should facilitate the reaction of O_2 with tetrahydropterin leading to a 4a-peroxy intermediate. This is because the direct reaction of triplet oxygen with singlet ground-state molecules is spin-forbidden (Hamilton, 1974), yet the $2e^-$ reduction of O_2 by reduced pterin (6,6,7,7-tetramethyltetrahydropterin) is a thermodynamically favorable process ($\Delta G° = -17$ kJ M^{-1}, pH 1; Eberlein et al., 1984).

If bound dioxygen retains some paramagnetic character, then reduction of O_2 may proceed through a radical-pair mechanism analogous to flavin-dependent O_2 activation. The spin-forbidden reaction of reduced flavin with O_2 is probably overcome by sequential one-electron transfers leading first to a flavin semiquinone–O_2^- radical pair that then collapses to form a 4a-hydroxperoxy flavin intermediate. This has been shown to be thermodynamically and kinetically feasible (Kemal et al., 1977; Anderson, 1982). However, the ease of forming a flavin semiquinone is in contrast to the great difficulty in forming a pterin semiquinone, which at pH 7.0 is at least 2 orders of magnitude less favorable on the basis of comproportionation data (Eberlein et al., 1984). Thus, another function of the metal ion in PAH may be to facilitate and (or) stabilize tetrahydropterin semiquinone formation.

In either of the above mechanisms of oxygen activation the close proximity of the Cu center to the pterin binding site would be required. In this regard, it has recently been demonstrated that $DMPH_4$ interacts directly with the cupric (Cu^{II}) ion in *C. violaceum* PAH through the N-5 position, although it is not known whether this interaction is maintained in the catalytically active cuprous (Cu^{I}) form of the enzyme (Pember et al., 1987). Further studies on the nature of the dioxygen interaction with PAH should help in understanding the pathway of oxygen activation.

Registry No. PAH, 9029-73-6; $DMPH_4$, 611-54-1; Phe, 63-91-2; O_2, 7782-44-7.

References

Anderson, R. F. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) p 278, Elsevier North-Holland, New York.

Ayling, J. E., & Bailey, S. W. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) p 294, Elsevier North-Holland, New York.

Ballou, D. P. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) p 301, Elsevier North-Holland, New York.

Bensadoun, A., & Weinstein, D. (1975) *Anal. Biochem. 70*, 241.

Bruice, T. C. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) p 265, Elsevier North-Holland, New York.

Cleland, W. W. (1970) *Enzymes (3rd Ed.) 2*, 1.

Cleland, W. W. (1975) *Biochemistry 14*, 3220.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Dix, T. A., Bollag, G., Domanico, P. L., & Benkovic, S. J. (1985) *Biochemistry 24*, 2955.

Detmar, K., & Massey, J. (1984) *J. Biol. Chem. 259*, 11265.

Dmitrienko, G. I., Snieckus, V., & Viswanatha, T. (1977) *Bioorg. Chem. 6*, 421.

Eberlein, G., Bruice, T. C., Lazarus, R. A., Henrie, R., & Benkovic, S. J. (1984) *J. Am. Chem. Soc. 106*, 7916.

Eickman, N. C., Solomon, E. I., Larrabee, J. A., Spiro, T. G., & Lerch, K. (1978) *J. Am. Chem. Soc. 100*, 6529.

Entsch, B., Ballou, D., & Massey, J. (1976) *J. Biol. Chem. 251*, 2550.

Evans, D. F. (1959) *J. Chem. Soc.*, 2003.

Fisher, D. B., Kirkwood, R., & Kaufman, S. (1972) *J. Biol. Chem. 247*, 5161.

Freedman, T. B., & Loehr, J. S. (1976) *J. Am. Chem. Soc. 98*, 2809.

Frieden, C. (1959) *J. Biol. Chem. 234*, 2891.

Gottschall, D. W., Dietrich, R. F., Benkovic, S. J., & Shiman, R. (1982) *J. Biol. Chem. 257*, 845.

Hamilton, G. A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) p 405, Academic Press, New York.

Karlin, K. D., Cruse, R. W., Gultneh, Y., Farooq, A., Hayes, J. C., & Zubieta, J. (1987) *J. Am. Chem. Soc. 109*, 2668.

Kaufman, S. (1975) in *Chemistry and Biology of Pteridines* (Pfleiderer, W., Ed.) p 291, de Gruyter, Berlin.

Kaufman, S., & Fisher, D. B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) p 285, Academic Press, New York.

Kemal, C., Chan, T. W., & Bruice, T. C. (1977) *J. Am. Chem. Soc. 99*, 7272.

Knott, G. D. (1979) *Comput. Programs Biomed. 10*, 271.

Krishnaswamy, P. R., Pamiljans, V., & Meister, A. (1962) *J. Biol. Chem. 237*, 2932.

Lazarus, R. A., Dietrich, R. F., Wallick, D. E., & Benkovic, S. J. (1981) *Biochemistry 20*, 6834.

Mager, H. I. X., Addink, R., & Berends, W. (1967) *Recl. Trav. Chim. Pays-Bays 86*, 833.

Marota, J. J. A., & Shiman, R. (1984) *Biochemistry 23*, 1303.
Massey, V., & Hemmerich, P. (1975) *Enzymes (3rd Ed.) 12*, 191.
Miller, M. R., McClure, D., & Shiman, R. (1975) *J. Biol. Chem. 250*, 1132.
Nakata, H., Yamauchi, T., & Fujisawa, H. (1979) *J. Biol. Chem. 254*, 1829.
Nielsen, K. H. (1969) *Eur. J. Biochem. 7*, 360.
Pauling, L., & Coryell, C. D. (1936) *Proc. Natl. Acad. Sci. U.S.A. 22*, 159.
Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1986) *Biochemistry 25*, 6611.
Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1987a) *Methods Enzymol. 142*, 50.
Pember, S. O., Benkovic, S. J., Villafranca, J. J., Pasenkiewicz-Gierula, M., & Antholine, W. E. (1987b) *Biochemistry 26*, 4477.
Phillips, W. D., & Poe, M. (1972) *Methods Enzymol. 24*, 304.
Rose, I. (1980) *Methods Enzymol. 64*, 47.
Rudolph, F. B., & Fromm, H. J. (1979) *Methods Enzymol. 63*, 138.
Shiman, R., & Gray, D. W. (1980) *J. Biol. Chem. 255*, 4793.
Simmons, M. G., & Wilson, L. J. (1978) *J. Chem. Soc., Chem. Commun. 498*, 634.
Solomon, E. I. (1981) *Met. Ions Biol. 3*, 41.
Thompson, J. S. (1984) *J. Am. Chem. Soc. 106*, 4057.
Van Vleck, J. H. (1932) in *Electric and Magnetic Susceptibilities*, p 228, Oxford University Press, Oxford, U.K.
Viola, R. E., & Cleland, W. W. (1982) *Methods Enzymol. 87*, 353.
Waalkes, T. P., & Undenfriend, S. (1957) *J. Lab. Clin. Med. 50*, 733.
Wallick, D. E., Bloom, L. M., Gaffney, B. J., & Benkovic, S. J. (1984) *Biochemistry 23*, 1295.
Weast, R. C., Ed. (1975) *CRC Handbook of Chemistry and Physics*, 56th ed., p E-125, CRC Press, Cleveland, OH.
Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., & Wilkinson, K. D. (1979) *Methods Enzymol. 62*, 185.

Effect of the Substitution Ala → Gly at Each of Five Residue Positions in the C-Peptide Helix†

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ABSTRACT: The substitution Ala → Gly has been studied in a unique-sequence peptide (related in sequence to the C-peptide of ribonuclease A) to determine its effect on C-peptide helicity at different residue positions. There is a substantial decrease in helicity for Ala → Gly at residue position 4, 5, or 6 but only a small decrease in helicity for Ala → Gly at end residue 1 and no decrease at end residue 13. The change for Ala → Gly is similar at position 4, 5, or 6; the change is caused chiefly by the difference in s, the helix growth parameter in the Zimm–Bragg model for α-helix formation, between Ala and Gly. Thus, the helicity of C-peptide depends sensitively on s at interior positions. The small change in helicity found for Ala → Gly at either end position suggests that the end residues are largely excluded from the helix, with the result that helicity is relatively unaffected by replacement of an end residue. Another possibility is that some helix-stabilizing effect is exerted by Gly only at an end position. Exclusion of an end residue from the helix might be caused either by fraying of the helix ends or by helix termination at an interior residue, resulting from a helix stop signal such as the Glu-2$^-$–Arg-10$^+$ salt bridge or the Phe-8–His-12$^+$ ring interaction.

The relative frequency (P_α) with which an amino acid occurs in protein α-helices can be correlated with s, the Zimm–Bragg helix growth parameter (Chou & Fasman, 1974). The correlation is, however, imperfect [see p 331 of Creighton (1984) and references cited therein]; Tyr has a considerably lower P_α value than would be expected on the basis of its value for s, whereas Gln, Ala, Leu, Lys$^+$, Asp$^-$, Glu$^-$, and Met all have higher P_α values than expected. It would be desirable to have an independent method of measuring the helix-stabilizing tendency of an amino acid, in addition to the host–guest method (Sueki et al., 1984), to investigate possible reasons for the deviations from an exact correlation.

The C-peptide of RNase A (a peptide containing the 13 N-terminal residues) provides a possible system for studying the deviations from an exact correlation. C-Peptide shows readily measured helicity in H_2O at 0 °C in a monomolecular reaction (Brown & Klee, 1971; Bierzynski et al., 1982), and derivatives of C-peptide have been found that are stronger helix formers (Shoemaker et al., 1987a). Since it has a unique sequence and contains several different amino acids, C-peptide provides a different type of helix-forming system than the random-sequence copolymers used in host–guest studies (Sueki et al., 1984). One aim of this paper is to test the suitability of C-peptide as a system in which to correlate the helix-stabilizing tendency of an amino acid with its frequency in α-helices in proteins. A second aim is to investigate the factors involved in a systematic study of position-dependent effects on helicity. The frequencies of the acidic and basic residues are quite different from each other at the N-terminus and, in an inverse manner, at the C-terminus of protein α-helices [Cook, 1967; see Chou and Fasman (1978) and references cited therein]. This effect may be explained by the helix-stabilizing interactions of these charged residues with the helix dipole (Ptitsyn & Finkelstein, 1970; Blagdon & Goodman, 1975; Shoemaker et al., 1987a), but some uncharged residues [e.g., Trp; Chou & Fasman, 1978] also show strikingly asymmetric patterns of frequency versus helix position, and

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