Mechanism of Metal-Independent Hydroxylation by *Chromobacterium violaceum* Phenylalanine Hydroxylase†

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ABSTRACT: Phenylalanine hydroxylase converts phenylalanine to tyrosine utilizing a tetrahydrobiopterin cofactor. Several key mechanistic questions have yet to be resolved, specifically the identity of the hydroxylating species and the role of the non-heme iron which is present in all of the mammalian PAHs. Recently, we have demonstrated that a bacterial PAH from *Chromobacterium violaceum* does not require any redox active metal for activity [Carr, R. T., & Benkovic, S. J. (1993) *Biochemistry 32*, 14132-14138]. To identify the function of iron in the mammalian PAH's, we have undertaken a series of experiments to compare the mechanisms of this metal-independent PAH with the iron-dependent PAH from rat liver. Using [4-^{2}H]phenylalanine as a substrate gave a kinetic isotope effect on hydroxylation of unity for CVPAH which is in agreement with previous values reported for RLPAH. The [4-^{2}H]phenylalanine underwent an NIH shift upon hydroxylation by CVPAH. The extent of deuterium retention at the 3-position of the tyrosine product was identical within experimental error for both RLPAH and CVPAH using [4-^{2}H]-phenylalanine and [2,3,5,6-^{2}H]phenylalanine as substrates. This suggests that PAH from either source probably does not directly mediate the NIH shift mechanism. No uncoupled pterin turnover was observed for CVPAH with either L-tyrosine or *p*-chloro-L-phenylalanine as substrate or tetrahydropterin as cofactor, each of which causes uncoupled turnover with RLPAH. CVPAH readily accepts 4-methylphenylalanine as a substrate giving 4-(hydroxymethyl)phenylalanine as the major product and 3-methyltyrosine as the only other minor product. The ratio of alkyl to aromatic hydroxylation is very close to the ratio previously obtained [Siegmund, H., & Kaufman, S. (1991) *J. Biol. Chem. 266*, 2903] for this analogue and two deuterated analogues. This suggests that both RLPAH and CVPAH utilize a very similar oxygenating intermediate. We also demonstrate that both metal-free and iron-dependent enzymes hydroxylate cyclohexylalanine in a stereoselective manner.

Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1, PAH[1]) catalyzes the transformation of L-phenylalanine (**1**) to L-tyrosine (**2**) with concomitant hydroxylation of a tetrahydropterin cofactor to a 4a-hydroxytetrahydropterin, utilizing molecular oxygen (see Figure 1).

In mammals this enzyme predominates in the liver, where it regulates the level of serum phenylalanine. If the activity of PAH is deficient, phenylalanine accumulates to toxic levels resulting in the clinical conditions of hyperphenylalaninemia and phenylketonuria. PAH, along with tyrosine hydroxylase (TH) and tryptophan hydroxylase (TpH), represents a class of pterin-dependent aromatic amino acid hydroxylases whose catalytic mechanisms remain undefined despite several decades of investigation [for review, see Shiman, (1985)].

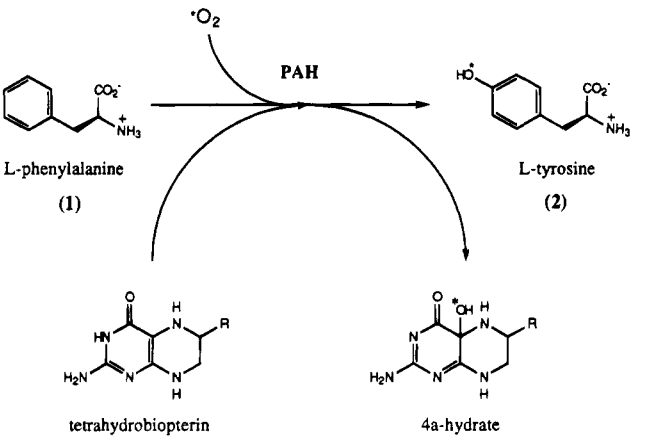

FIGURE 1: Hydroxylation of L-phenylalanine catalyzed by CVPAH.

A central mechanistic question is the manner in which triplet oxygen combines with the enzyme and substrates to form an activated singlet oxygen intermediate capable of aromatic hydroxylations. A pterin 4a-hydroperoxide has been proposed to be this intermediate [based on the analogy with 4a-hydroperoxy flavins responsible for the hydroxylation of certain aromatic compounds in bacterial systems (Massey & Hemerich, 1976; Beaty & Ballou, 1980)]. A related "oxenoid" intermediate formed by opening of the

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[1] Abbreviations: 4aOHDMPH$_4$, 4a-hydroxy-6,7-dimethyltetrahydropterin; BH$_4$, tetrahydrobiopterin; CVPAH, *Chromobacterium violaceum* phenylalanine hydroxylase; DMPH$_2$, 6,7-dimethyldihydropterin; DMPH$_4$, 6,7-dimethyltetrahydropterin; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; MCPBA, *meta*-chloroperbenzoic acid; 6-MPH$_4$, 6-methyltetrahydropterin; NMR, nuclear magnetic resonance; RLPAH, rat liver phenylalanine hydroxylase; TFA, trifluoroacetic acid; TH, tyrosine hydroxylase; TpH, tryptophan hydroxylase.

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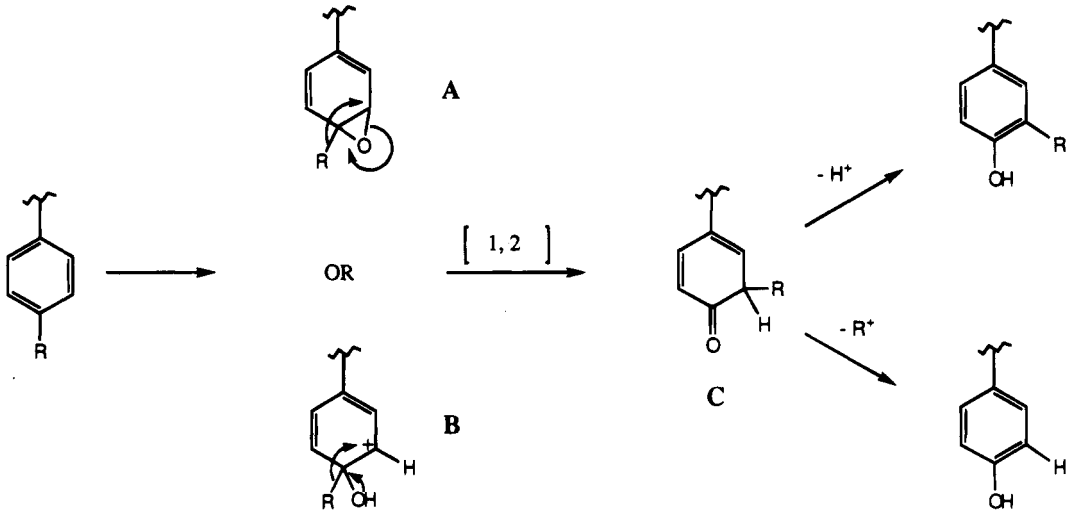
Scheme 1. NIH Shift Mechanism, Showing 1,2 Shift of *para* Substituent



pyrazine ring of the hydroperoxide has been proposed by Hamilton (1974) on the basis of its more potent oxygen donating ability.

Non-heme iron was found in stoichiometric amounts in rat liver PAH (RLPAH) (Fisher et al., 1972). This iron must be present in its reduced form (Fe^{2+}) for catalytic activity (Gottschall et al., 1982). An iron-μ-oxo-pterin hydroperoxide was proposed by Dix and Benkovic (1985) on the basis of the ability of substrate analogs to partially uncouple pterin oxidation from substrate hydroxylation and the pterin oxidase activity of PAH in the presence of various peroxides and peracids. A hypervalent "ferryl" (Fe^{IV}=O) ion has also been proposed as a potent oxygen source (Dix & Benkovic, 1988) in analogy to the (Fe^{V}=O) ion thought to be the hydroxylating agent of cytochrome P-450 enzymes (White & Coon, 1980; Guengerich, 1990).

To better understand the role of the metal center in PAH, we have studied the bacterial PAH from *Chromobacterium violaceum* (CVPAH). This enzyme, a 33 kDa monomer, has an overall 24% sequence homology to the catalytic domain of the RLPAH subunit, with greater homology in certain regions highly conserved among all known pterin-dependent hydroxylases (Onishi et al., 1991). This enzyme was found to contain 1 mol of copper as copper(II) after dialysis against 0.1 M NaCl and 80 mM HEPES, pH 7.4 (Pember et al., 1986). At that time it was believed that the copper was required and would provide the same functionality as the iron in mammalian PAHs. Recently, however, we have prepared "metal-free" CVPAH and found that it is active without copper (Carr & Benkovic, 1993). Copper (II) was shown to be an inhibitor of CVPAH and must be removed from the enzyme for activity. Iron, cobalt, nickel, molybdenum, and several other metals were also shown to be inhibitory and are absent in the "metal-free" enzyme preparation. Therefore, we have concluded that CVPAH does not require any metal cofactor in its chemical mechanism. This would of course eliminate any metal−oxo species as the activated oxygen intermediate for this enzyme.

The question that arises is whether the bacterial and mammalian enzymes share a common mechanism, which would exclude a mechanistic role for the iron in mammalian systems, or has an iron-dependent mechanism in mammals and a metal-independent mechanism in bacteria evolved separately? If the latter is true, a comparison of mechanistic data between mammalian PAH and CVPAH should yield important differences that could lead to an explanation of the role of iron in the mechanism of mammalian pterin-dependent hydroxylases.

There is a considerable body of research regarding the mechanism of mammalian pterin-dependent monooxygenases, especially RLPAH. Most of these studies involved altered amino acid substrates or pterin cofactors to probe various aspects of the mechanism. Guroff et al. (1966, 1967) showed that tritium substituted at the 4-position of phenylalanine was not lost upon hydroxylation at that position by RLPAH, but instead most of the product contained tritium at the 3-position. This has become known as the NIH shift. A mechanism proposed for this shift, depicted in Scheme 1, has the C(4) or *para* substituent migrating to the 3-position upon formation of the C-3 carbocation (shown as B in Scheme 1). A protium or deuterium ion is then lost from C-3 on rearomatization of the phenyl ring. This shift has also been observed in nonenzymatic model reactions of phenylalanine hydroxylations (Castle et al., 1978; Kumar & Murray, 1984). No deuterium kinetic isotope effect is observed in the reaction of RLPAH with [4-^{2}H]L-phenylalanine when BH_4 is used as the cofactor, and only a slight effect of 1.3 is observed with the synthetic 6-MPH_4 cofactor (Abita et al., 1984). Recently an ^{18}O V_{max}/K_m kinetic isotope effect of 1.018 was determined for tyrosine hydroxylase (Fitzpatrick, 1991). These results along with the study of Bailey et al. (1991), which showed that the V_{max} of the RLPAH reaction increased linearly with the autoxidation rate of various C-6 substituted pterins, suggest that the initial formation of an activated oxygen−pterin intermediate is rate determining.

Another use of substrate analogues has been in the study of "uncoupled" turnover, where the cofactor is oxidized at a rate greater than the rate of substrate hydroxylation. It was noted by Dix and Benkovic (1985) that using either *p*-chlorophenylalanine as a substrate or unsubstituted tetrahydropterin as the cofactor partially uncoupled the RLPAH reaction. In their study roughly three pterin molecules were oxidized for every substrate molecule hydroxylated. Kaufman has reported completely uncoupled pterin oxidation with lysolecithin activated RLPAH when using tyrosine as a

substrate (Davis & Kaufman, 1993).

In this study we extend these mechanistic studies to CVPAH with the addition of a novel aliphatic hydroxylation reaction, in order to directly compare the mechanism of iron-dependent and metal-independent hydroxylation. The similarities and differences between these two forms of PAH are discussed, leading to a general hypothesis for the role of iron in the mammalian pterin-dependent hydroxylases.

EXPERIMENTAL PROCEDURES

Materials

L-Phenylalanine, catalase, 4-chloro-L-phenylalanine, and HEPES were from Sigma. HPLC grade solvents were from Fisher Scientific. Chemicals for synthesis were obtained from Aldrich. The concentrated HEPES buffer was passed through a 25 cm × 1 cm column of Chelex 100 (Bio-Rad) to remove trace metals. $DMPH_4$ was synthesized by the procedure of Mager et al. (1967) with catalytic reduction over platinum. 4-Methyl-L-phenylalanine (**3**) was from Schweitzerhall. L-Phenylalanine and all phenylalanine analogues were purified by reversed-phase HPLC on a Waters 600E system with either a 1 cm (Whatman) or 0.25 cm ODS column (Phenomenex), prior to enzyme assays. This was necessary to remove unidentified inhibitory agents (perhaps metals) from both the synthetic and commercially available stocks. Elution was isocratic with either 100% water or 5% acetonitrile in water at 3 mL min^{-1} for the Whatman column and 0.4 mL min^{-1} for the Phenomenex column. Recombinant RLPAH produced with baculovirus in Sf-9 cells (Gibbs et al., 1993) was a generous gift of Dr. Barbara Gibbs. 4-Hydroxymethyl-L-phenylalanine (**4**), 3-methyl-L-tyrosine (**5**), 4-methyl-3-hydroxy-L-phenylalanine (**6**), 2-amino-[3-$^2H_2$]-((4-[$^2H_3$]methyl)phenylpropanoic acid) (**7**), and 2-amino-[3-$^2H_2$](4-[$^2H_3$]methyl-[2,3,5,6-$^2H_4$]phenyl-propanoic acid) (**8**) were generous gifts from Dr. Seymour Kaufman (see Figure 3). NMR spectra were taken on Bruker ACE-200, AM-360 (^1H 360 MHz, ^{13}C 90.54 MHz), and AM-500 (^{13}C 125.76 MHz) instruments. FAB mass spectra were collected on a Kratos MS-50 using a glycerol matrix with xenon ionization; EI and CI mass spectra were taken on a Kratos MS 9/50 with isobutane ionization.

Synthesis

[4-^2H]L-Phenylalanine (**9**). 4-L-Bromophenylalanine (Sigma) was reduced with 6% sodium amalgam in ^2H_2O to give racemic [4-^2H]phenylalanine in quantitative yield. The mixture of L- and D-[4-^2H]phenylalanine was N-acylated by treatment with acetic anhydride and pyridine in 65% yield. The L- isomer was then resolved by enantioselective deacylation with *Aspergillus melleus* acylase I (Sigma). The optical purity of **9** was confirmed by the acylase I reaction which proceeded to exactly 50 ± 2% product by HPLC. In addition, **9** was completely turned over by CVPAH by HPLC assay, whereas D-phenylalanine (Sigma) was found not to be turned over by CVPAH under the same conditions with a detection limit of <2%. δ_H (^2H_2O, 200 MHz) 3.05−3.35 (2H, m, CH_2), 3.98 (1H, dd, J = 5.2, 8 Hz, CH), 7.32 (2H, A_2X_2 d, J = 7.9 Hz, aromatic), 7.42 (2H, A_2X_2 d, J = 7.9 Hz, aromatic). E. I. M/S (bis-TMS derivative) 295 ([M − CH_3]$^+$, 1%), 267 ([M − CO_2 + H]$^+$, 3%), 218 ([M − C_6H_4-DCH_2]$^+$, 26%), 193 ([M − COOTMS]$^+$, 20%), 121 ([CH_6H_4-DCH_2CHNH_2]$^+$, 100%), 92 ([M − CH(NHTMS)COOTMS]$^+$, 17%). HPLC retention time was 14 min on the Whatman column. By mass spectrometry **9** contained 99 ± 1% deuterium. The A_2X_2 pattern in the aromatic region of the ^1H NMR spectrum confirmed exclusive ring deuteriation in the 4-position.

[2,3,5,6-$^2H_4$]L-Phenylalanine (**11**). [2,3,4,5,6-$^2H_5$]L-phenylalanine (**10**) (Cambridge Isotopes) was brominated by the vapor diffusion method of Brundish and Wade (1976) using neat bromine. 4-Bromo-[2,3,5,6-$^2H_4$]L-phenylalanine was isolated from the mixture of brominated products by reverse-phase HPLC in 15% yield, using commercially available 4-bromophenylalanine (Sigma) as an HPLC standard. **11** was obtained by reduction with 6% sodium amalgam in H_2O. δ_H (^2H_2O, 200 MHz) 3.07−3.45 (2H, m, CH_2), 3.94 (1H, dd, J = 5, 7.2 Hz, CH), 7.45 (1H, s, aromatic). E. I. M/S gave the same fragmentation pattern as described above for **9**, with peaks 3 mass units higher. HPLC retention time was 14 min. By mass spectrometry **11** contained 97 ± 2% tetradeuteriated species. The observation of a single aromatic peak in the ^1H NMR spectrum of **11** is consistent with the presence of a unique aromatic proton in the 4-position.

L-*Cyclohexylalanine* (**12**). L-Tyrosine (2 g) was reduced over 500 mg of platinum on charcoal (5%) in 25 mL of 1 M HCl as described by Bumpus (1974). Crystallization from methanol/diethyl ether gave a white solid (1.68 g, 85%) that was further purified by HPLC (with 5% acetonitrile in water). mp 234−236 °C (lit. 248−250 °C); Acc. M/S (EI) observed M^+ 171.1244, calcd for $C_9H_{18}NO_2$ 171.1254; δ_H (360 MHz, $C^2H_3O^2H$) 0.96 (2H, m, C-7 H_2), 1.28 (4H, m, C-6 and C-8 H_2), 1.55 (1H, m, C-4 H), 1.73 (6H, m, C-3, -5 and -9 H_2) and 3.97 (1H, dd, J 6.27 and 7.91 Hz, C-2 H); δ_C (125.76 MHz, ^2H_2O) 26.71−27.92 (C-6, -7 and -8), 33.63 (C-4), 34.17 and 34.73 (C-5 and 9), 39.36 (C-3), 51.78 (C-2) and 172.45 (C-1); FAB M/S 172 (M + H)$^+$.

[2,2,3,3,4,4,5,5,6,6,7-$^2H_{11}$]L-Cyclohexylalanine (**13**). [2,3,-4,5,6-$^2H_5$]L-phenylalanine (Sigma) (100 mg) was reduced over platinum on charcoal (50 mg) with deuterium gas in 10 mL of 0.1 M DCl. Purification was carried out exactly as for the protiated analogue. δ_H (360 MHz, $C^2H_3O^2H$) 1.67 (1H, AB dd, J 14.3 and 6.4 Hz, 1 of CH_2CHNH_2), 1.81 (1H, AB dd, J 14.3 and 7.75 Hz, 1 of CH_2CHNH_2) and 3.94 (1H, dd, J 7.75 and 6.4 Hz); δ_C (125.76 MHz, ^2H_2O) 25.2−26.54 (m), 32.89 (m), 33.91 (m), and 172.71; FAB M/S 183 (M + H)$^+$.

Enzyme Preparation

CVPAH was grown in *E. coli* as described previously (Onishi et al., 1991). It was purified and made "metal-free" by extraction of copper with DTT as described by Carr and Benkovic (1993). All of the CVPAH enzyme used in this study was greater than 95% pure as judged by SDS−PAGE and had specific activity greater than 10 μmol min^{-1} mg^{-1} with less than 0.05 mol of copper per mol of enzyme as measured by atomic absorption spectrophotometry.

Enzyme Assays

CVPAH activity was assayed by following the change in absorbance at 275 nm (Miller et al., 1978; Pember et al., 1987) using a $\Delta\epsilon$ of 2600 M^{-1} cm^{-1} for phenylalanine turnover (Carr & Benkovic, 1993) and 900 M^{-1} cm^{-1} for cyclohexylalanine turnover. The normal assay solution

contained 30 mM HEPES buffer, pH 7.4, 180 μM $DMPH_4$, 6.0 mM DTT, 1.0 mM L-phenylalanine, and 90 μg/mL catalase with 50−200 nM CVPAH. CVPAH concentration was determined by the BCA (Pierce) method. For detecting the 4a-hydroxypterin product the assay was run in 8 mM Tris buffer at pH 8.45.

NIH Shift Experiments

The [4-^{2}H]L-phenylalanine and [2,3,5,6-$^2H_4$]L-phenylalanine substrates were turned over by PAH under the normal assay conditions described above but carried out on a 5 mL scale. The protein was precipitated with 1, 2-dichloroethane and then removed by centrifugation. The aqueous portion was injection onto the ODS HPLC column, and the L-tyrosine reaction product was isolated by isocratic elution with 100% H_2O at a flow rate of 1 mL/min. Tyrosine eluted as a single peak with a λ_{max} of 275 nm and a retention time of 9.5 min. The tyrosine fraction was evaporated *in vacuo* (Speed Vac) before silylation with 2−3 drops of a mixture of hexamethyldisilazone/trimethylchlorosilane and pyridine (Supelco kit). After 30 min at room temperature the reaction mixture was directly injected onto the electron impact mass spectrometer, in order to evaluate the isotopic content. The mass spectra of TMS derivatized tyrosine generally contained a mixture of tri- and di-silylated species. The fragmentation pattern given below for monodeuteriated tyrosine is typical: 383 ([M(trisilylated) − CH_3]$^+$, 0.5%), 355 ([M − CO_2]$^+$, 0.5%), 326 ([M(disilylated)]$^+$, 1.1%), 311 ([M(disilylated) − CH_3]$^+$, 6.7%), 218 ([M − CH_2-C_6H_3D-OSi(CH_3)$_3$]$^+$, 18%), 180 ([M − (CHNHTMS)COOTMS]$^+$, 100%). Isotopic ratios were calculated using the stable [M − (CHNHTMS)COOTMS]$^+$ species (peak at 180 for monodeuteriated species), correcting for any "−1" peak intensity present in the unlabeled standard. The quoted values for deuterium retention are the average values obtained from at least three independent sample injections onto the mass spectrometer.

The isotopic content of labeled phenylalanines was evaluated by essentially the same mass spectrometric analysis. For phenylalanine, however, the stable $C_6H_4DCH_2CHNH_2$ species (peak at 121 for monodeuteriated species) was used in isotope ratio calculations (fragmentation pattern given in synthetic section).

"Uncoupled" Turnover

To detect possible "uncoupled" pterin oxidation the above assay was done substituting 4-chloro-L-phenylalanine (2.0 mM) and L-tyrosine (1.05 mM) for the L-phenylalanine substrate or tetrahydropterin (180 μM) for the $DMPH_4$ cofactor. H_2O_2 was assayed in these solutions using HRP and 4-methoxy-α-naphthol (Guilbault & Kramer, 1964). The pterin oxidase activity of CVPAH was examined using 40 μM H_2O_2 or 40 μM MCPBA in the presence of 2.0 μM CVPAH and 200 μM $DMPH_4$ in 30 mM HEPES buffer. Spectra were collected every minute to detect formation of oxidized $DMPH_4$. This reaction was compared to a control omitting CVPAH.

Reaction with 4-Methyl-L-Phenylalanine

The products of the 4-methylphenylalanine reaction were isolated, identified, and quantitated by HPLC. The reactions were run in 8 mM Tris buffer, pH 8.45, saturated with oxygen at 25 °C with 6.0 mM cysteine, 180 μM $DMPH_4$, 90 μg/mL catalase, 2 mM 4-methylphenylalanine, and 1.0 μM CVPAH. When RLPAH was used, 150 μM 6-MPH_4 was substituted for the $DMPH_4$. These reactions were monitored by spectral changes from 240 to 400 nm with scans recorded every 2 min. The protein was removed from the samples by precipitation with 1,2-dichloroethane followed by centrifugation. Aliquots of 100 μL were injected onto a 0.25 by 25 cm C-18 column (Phenomenex) and eluted isocratically with 100% water. Two peaks not present in control reactions lacking enzyme or pterin were observed. The major peak was collected and identified by FAB M/S and NMR. The minor peak was identified by co-elution with authentic standards. Quantitation was by integration of the product peaks and comparison with standard curves of the authentic standards. The deuterated analogues were quantitated by this same procedure.

L-Cyclohexylalanine as a PAH Substrate

The reaction of CVPAH with cyclohexylalanine was monitored by spectral scans from 240 to 400 nm. Since there is little change in absorbance with aliphatic hydroxylation, most of the changes are due to pterin oxidation. The assay contained 30 mM HEPES buffer, pH 7.4, 6.0 mM DTT, 180 μM $DMPH_4$, 90 μg/mL catalase, 2.0 mM L-cyclohexylalanine, and 1 μM CVPAH. The reaction produced a new ninhydrin active spot on cellulose TLC plates developed with 5/4/2.6/.4 (pyridine/butanol/water/acetic acid). This material was isolated from a large (50 mL) scale reaction by HPLC. After removal of the protein by ultrafiltration (Centriprep 10, Amicon) and concentration to 10 mL by evaporation, 0.5 mL aliquots were injected onto a 1 by 30 cm reversed-phase column (Whatman). Elution was isocratic with 100% water with UV detection at 220 nm. The peak at a RT of 10 min was collected and evaporated to dryness. This product was identified by ^{1}H and ^{13}C NMR and positive ion FAB mass spectroscopy. δ_H (360 MHz, ^2H_2O) 1.1−1.9 (11H, m, ring + β-methylene hydrogens), 3.42 (0.9H, m, C*H*OH, axial OH), 3.58 (0.1H, dd, *J* 5.5 and 3.2 Hz, C*H*OH, equatorial OH) and 3.76 (1H, m., C*H*NH_2). δ_C (90.54 MHz, ^2H_2O) (relative to dioxane at 67.6 ppm) 23.46, 24.32, 28.25, 28.16, 29.14, 34.39, 50.44, 67.79, and 70.12 (carbon bearing OH in axial and equatorial orientations) and 171.12. FAB (positive ion) 188 (M + H)$^+$. The stereoselectivity of hydroxylation was assessed by quantitation of the relative integrals of the peaks in the ^{1}H NMR spectrum arising from the proton on the carbon atom bearing the hydroxyl group.

Coupled Turnover with L-Cyclohexylalanine

The same assay mixture was used as for the turnover experiments except that only 100 μmol of L-cyclohexylalanine was used. Hydroxylation of this oxidized 90 ± 10 μmol of $DMPH_4$, using $\Delta\epsilon_{278} = 1200\ M^{-1}\ cm^{-1}$ (Carr & Benkovic, 1993).

RESULTS

NIH Shift. [4-^{2}H]-L-Phenylalanine (**9**) was converted to tyrosine product by both CVPAH and by RLPAH. In each case the tyrosine product was isolated by HPLC then silylated and analyzed for isotopic content by mass spectrometry. The isotopic content of **9** was also determined by mass spectrometry. Substrate (**9**) was determined to contain 99 ± 1% atomic deuterium by mass spectrometry, and all of the

Table 1: Deuterium Retention in Turnover of Deuteriated L-Phenylalanine Analogues

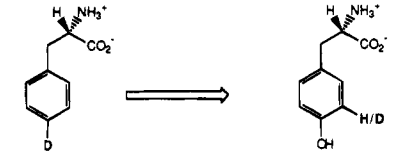
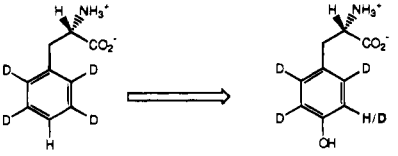
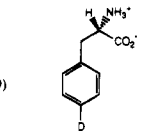
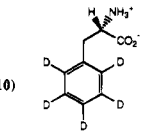
		Deuterium content of product
(9)		CV: 85 ± 2 % (d^1) RL: 84 ± 2 % (d^1)
(11)		CV: 84 ± 2 % (d^4) RL: 84 ± 2 % (d^4)

Table 2: Substrate Isotope Effects for CVPAH

substrate	V^D	$(V/K)^D$
(9) 	1.01 ± 0.1	1.05 ± 0.1
(10) 	0.95 ± 0.1	1.02 ± 0.1

deuterium appeared to be in the 4-position on the basis of the coupling pattern in the ^{1}H NMR spectrum. The tyrosine products had retained 85 ± 2% and 84 ± 2% deuterium for the CVPAH and RLPAH turnover experiments, respectively (Table 1). [2, 3, 5, 6-$^2H_4$]L-phenylalanine (**11**) was also turned over by both CVPAH and RLPAH, and the products were analyzed for isotopic content. [2, 3, 5, 6-$^2H_4$]L-Phenylalanine (**11**) contained 97 ± 2% d_4 species whereas the products (**12**) contained 84 ± 2% d_4 species for both the CVPAH and RLPAH reactions (Table 1), the remaining being predominantly d_3 species.

Kinetic Isotope Effects. The deuterium kinetic isotope effects on V and V/K were determined for the CVPAH reaction using the labeled substrates [4-^{2}H]L-phenylalanine (**9**) and also for [2, 3, 4, 5, 6-$^2H_5$]L-phenylalanine (**10**). The deuterium kinetic isotope effects were determined to be unity within experimental error for both **9** and **10** (Table 2).

Reaction with 4-Methylphenylalanine. Two products were found in the reaction of CVPAH with 4-methylphenylalanine. The major product was identified as 4-hydroxymethylphenylalanine (**4**), and the minor product was 3-methyltyrosine (**5**), as shown in Figure 2. The ratio of these products is given in Table 3 along with the values reported previously for RLPAH by Siegmund and Kaufman (1991). In both cases hydroxylation of the methyl group is favored over hydroxylation of the aromatic ring. We were unable to detect any 3-hydroxy-4-methylphenylalanine (**6**), which would be produced by hydroxylation of this substrate directly at the 3-position. When the methyl group of 4-methylphenylalanine is deuteriated or when both the methyl group and the

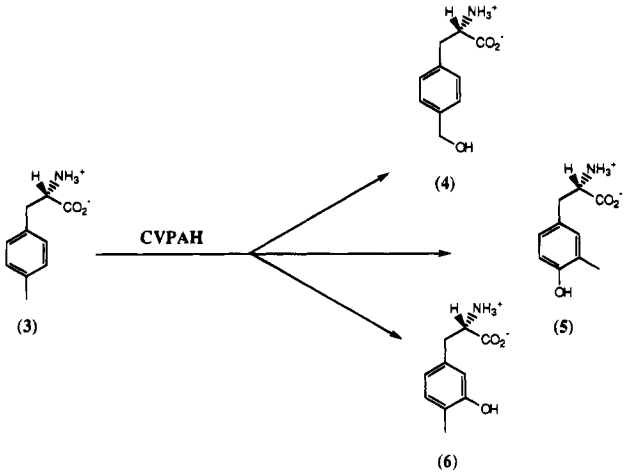


FIGURE 2: Turnover of 4-methyl-L-phenylalanine.

Table 3: Regioselectivity in Hydroxylation of l-Phenylalanine Analogues by CVPAH and RLPAH

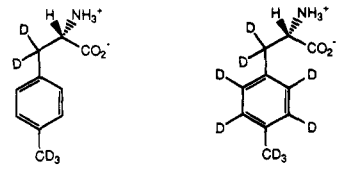
	CVPAH, position hydroxylated (%)			RLPAH, position hydroxylated[a] (%)		
substrate	methyl	*para*	*meta*	methyl	*para*	*meta*
4-methyl-L-Phe	74	26	0	79	21	0
(**7**)	27	73	0	22	78	0
(**8**)	13	87	0	20	80	0

[a] Data from Siegmund and Kaufman (1991).

phenyl ring is deuteriated (compounds **7** and **8**, respectively), the amount of alkyl hydroxylation is decreased relative to aromatic hydroxylation as seen in Table 3. Again, this is in agreement with the previous results for RLPAH (Siegmund & Kaufman, 1991).

Uncoupled Turnover. A standard CVPAH assay mixture was set up except that 4-chloro-L-phenylalanine was substituted for L-phenylalanine. Under these conditions no hydroxylation of 4-chloro-L-phenylalanine was detected and no oxidation of $DMPH_4$ cofactor to the quinonoid form above background was observed (less than 5% above background). No H_2O_2 could be detected in this reaction by the peroxidase assay used. 4-Chloro-L-phenylalanine reversibly inhibits CVPAH with an IC_{50} of 200 μM. Tyrosine does not produce any coupled or uncoupled turnover with CVPAH using $DMPH_4$ as a cofactor and does not inhibit the reaction with phenylalanine at concentrations up to 1.5 mM. Tetrahydropterin is a cofactor of CVPAH, but the reaction appears tightly coupled with a stoichiometry of 1 ± 0.1 mol of tyrosine produced per mol of tetrahydropterin oxidized. Unlike RLPAH there is no pterin oxidase activity of CVPAH in the presence of H_2O_2, *tert*-butylhydroperoxide or MCPBA.

Cyclohexylalanine Hydroxylation. L-Cyclohexylalanine (**12**) was tested as a substrate for PAH as outlined under Experimental Procedures, the reaction being monitored by UV absorbance changes due to pterin oxidation. The spectral changes of the cyclohexylalanine reaction in Tris buffer at pH 8.45 are shown in Figure 4. They are identical for both RLPAH and CVPAH. 4a-Hydroxy-$DMPH_4$ is identified by the absorbance increase between 240 and 265 nm. The increases in absorbance at 275 and 340 nm are indicative of the 7,8-$DMPH_2$ which is produced by a DTT catalyzed tautomerization of the quinonoid $DMPH_2$ (Carr & Benkovic, 1993). A compound of elution time on reverse-phase HPLC of 10 min was identified and characterized (see Experimental



(7) (8)

FIGURE 3: 2-Amino-[3-$^2H_2$]((4-[$^2H_3$]methyl)phenylpropanoic acid) (**7**) and 2-amino-[3-$^2H_2$](4-[$^2H_3$]methyl[2,3,5,6-$^2H_4$]phenylpropanoic acid) (**8**).

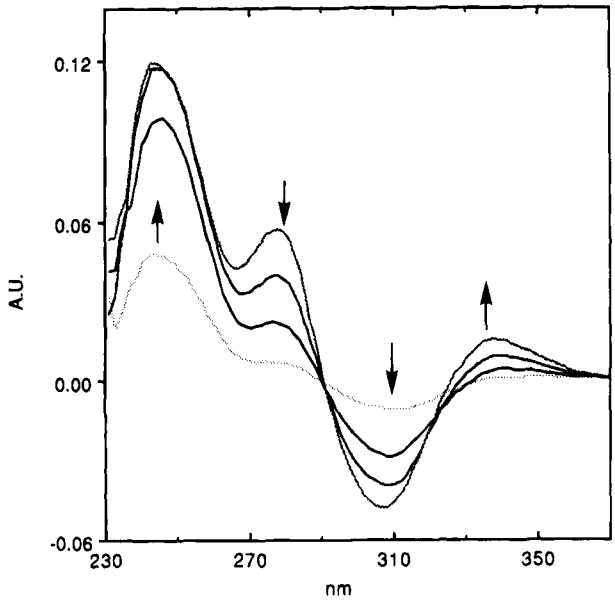


FIGURE 4: Spectral changes on oxidation of L-cyclohexylalanine by CVPAH. Scans were acquired at 1 min intervals. The increases in absorbance at 275 and 335 nm are due to 7,8-$DMPH_2$ formation. The decrease in absorbance at 305 nm is due to loss of $DMPH_4$. The initial increase in absorbance at 245 nm arises from the formation of 4aOH $DMPH_4$.

Procedures) as L-cyclohexylalanine hydroxylated at the "*para*" or C-7 position of the ring.

There are two possible stereochemistries for hydroxylation of the cyclohexyl ring, axial and equatorial. By comparison with spectra of known compounds (Pretsch et al., 1989), the stereochemistry of hydroxylation at C-7 was identified as about 90% axial (signal at 3.42 ppm in the ^1H NMR) and 10% equatorial (signal at 3.58 ppm). The bulky alanine fragment is assumed always to adopt the equatorial position on the cyclohexyl ring (see Figure 5).

The reaction was determined to be completely coupled by the quantitation of pterin oxidized using limiting concentrations of L-cyclohexylalanine (100 μM). The measured values of K_m and V_{max} were 300 mM and 2.4 μmol min^{-1} mg^{-1}, respectively, for the reaction of CVPAH (only 4 times slower than with L-phenylalanine as substrate). The activity of RLPAH was ca. 50% less than that for CVPAH under the conditions of the assays (HEPES, pH 7.4, with $DMPH_4$ cofactor). The product was identified my comigration on HPLC with an authentic sample isolated from the CVPAH turnnover. The ratio of axial to equatorial hydroxylation was not quantitated.

DISCUSSION

Any chemical mechanism of pterin-dependent hydroxylases must account for the NIH shift. While addition of a radical oxygen species to phenylalanine is not ruled out by the observation of an NIH shift, the evidence from chemical models of the PAH reaction favors an electrophilic attack on the phenyl ring by the oxygenating intermediate followed by a 1,2-hydride shift and rearomatization (Dix & Benkovic, 1988). We report here the observation of an NIH shift in the hydroxylation of [4-^2H]phenylalanine by "metal-free" CVPAH. We have found that the extent of the NIH shift for [4-^2H]phenylalanine is the same, within experimental error, for both RLPAH and CVPAH. This suggests that the chemistry subsequent to initial addition of oxygen to phenylalanine proceeds through a common mechanism in both the iron-requiring RLPAH and the metal-independent CVPAH.

Does either enzyme play any role in the hydride shift or rearomatization steps that constitute the NIH shift? The NIH shift might proceed by a stereospecific migration of a hydride (deuteride) followed by preferential proton (deuteron) loss from one face of the phenyl ring to give the observed mixture of isotopes at the 3-position. We have found that for [2,3,5,6-$^2H_4$]L-phenylalanine, where a ^1H migrates, the extent of deuterium retention was the same as for [4-^2H]L-phenylalanine, where a ^2H migrates. This implies that there is no stereoselectivity in the proton (deuteron) loss on rearomatization, i.e., isotopic discrimination does not involve a stereoselective loss of the nonmigrating hydrogen isotope. The results for the extent of deuterium retention for this substrate are the same for either CVPAH or RLPAH and are in agreement with the results of Guroff et al. (1967) and Guroff and Rhoades (1967), who performed a similar experiment with PAH from *Pseudomonas sp.*

The evidence suggests that the isotopic discrimination results from a kinetic isotope effect with preferential cleavage of a C−H bond rather than a C−D or C−T bond. The 85% retention of deuterium during the rearomatization step results from an associated isotope effect of 5.5 ± 0.8 (Table 1). This value is gratifyingly close to the published values of 5.6 obtained for the analogous rearomatizations during the iodination of 4-nitrophenol carried out in aqueous solvent (Grovenstein & Aprahamian, 1962) and 4.8−6.5 in the diazotization of a range of substituted naphthols (Gold, 1964). This is also in agreement with the greater degree of tritium retention compared to deuterium when [4-^3H]L-phenylalanine is used as the substrate. Thus it is altogether possible that the rearomatization leading to tyrosine occurs after dissociation of an intermediate (A, B, or C in Scheme 1) from the enzyme.

The lack of any kinetic isotope effect is consistent with some event prior to the hydride shift being rate determining. These experiments were done using $DMPH_4$ which gives the fastest turnover with this enzyme. In the case of RLPAH there is also no deuterium isotope effect observed with the natural biopterin cofactor and only a slight effect of 1.3 ± 0.1 was found with the synthetic 6-MPH_4 (Abita, 1984). This is in agreement with the results of Bailey et al. (1991), who demonstrated a correlation of the catalytic rate of RLPAH with the rate at which various pterin cofactors undergo single electron transfer to oxygen, thus implicating the formation of an oxygen−pterin intermediate as the overall rate-limiting step of the reaction. This is also consistent with the finding of an ^{18}O V/K_m kinetic isotope effect of 1.018 by Fitzpatrick (1991) with the closely related tyrosine hydroxylase, implicating the initial formation of a pterin−oxygen adduct as the rate-limiting step in TH catalysis.

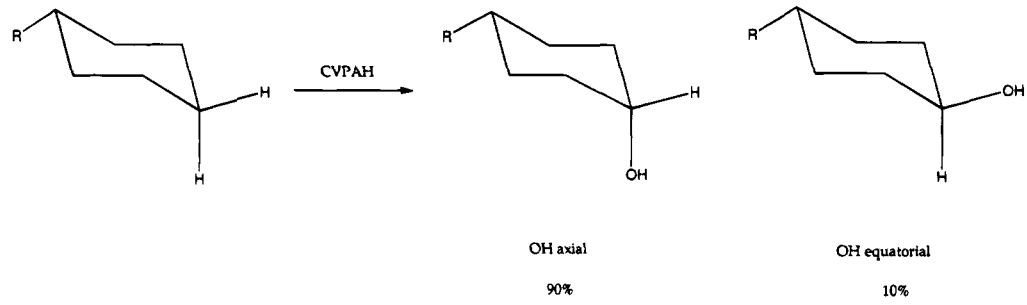


FIGURE 5: Product ratio from hydroxylation of the cyclohexyl ring of L-cyclohexylalanine. R = H_2NCHCO_2H.

Since the iron of mammalian PAH does not appear to be involved in the mechanism subsequent to the addition of oxygen to the phenylalanine ring, we sought experiments that would compare the mechanisms of the initial addition of oxygen to the phenylalanine ring for both iron-dependent (RLPAH) and metal-independent (CVPAH) hydroxylases. Little is known about the nature of the hydroxylating intermediate, but it is known that it is capable of both aromatic and alkyl hydroxylations. This was first shown by Guroff et al. (1967), who demonstrated that PAH from *Pseudomonas* hydroxylated 4-methyl-L-phenylalanine both on the aromatic ring and at the benzylic position. This study has recently been extended by Siegmund and Kaufman (1991), who demonstrated that the two products of the reaction of RLPAH (Figure 2) were 3-methyl-L-tyrosine, the expected NIH shifted product, and 4-hydroxymethyl-L-phenylalanine. The major product was in fact the 4-hydroxymethyl-L-phenylalanine (Table 3). When deuterium was substituted for the methyl hydrogens, the product ratio shifted in favor of the aromatic hydroxylation. A hypervalent iron−oxygen species would be the most likely intermediate that would perform both types of hydroxylations.

We report here that "metal-free" CVPAH does indeed hydroxylate 4-methyl-L-phenylalanine on the benzylic methyl group. Furthermore, the product ratio of 4-hydroxymethyl-L-phenylalanine to 3-methyl-L-tyrosine is identical, within experimental error, to the ratios reported by Siegmund and Kaufman (1991) (Table 3). The partitioning of products for deuterium substituted 4-methyl-L-phenylalanine substrates are also in good agreement. Collectively, the data suggest the hydroxylating intermediates for both iron-dependent RLPAH and metal-independent CVPAH are similar if not identical. Since the CVPAH enzyme has no metal dependence, this would argue against an iron−oxo species being the hydroxylating intermediate common to both enzyme forms. We also tested several analogues where the alkyl hydroxylation would be at a relatively unactivated carbon (as opposed to the benzylic methyl of 4-methyl-L-phenylalanine). It has already been reported that RLPAH does hydroxylate the unactivated ϵ-carbon of L-norleucine (Kaufman & Mason, 1982). We found that both CVPAH and RLPAH hydroxylated L-cyclohexylalanine, the fully ring reduced phenylalanine analogue. The only product detected from this reaction was 4-hydroxy-L-cyclohexylalanine, with 90% axial hydroxylation. The observation of stereoselective hydroxylation in a pterin-dependent enzyme system had not been made prior to these experiments and suggests that the hydroxylating intermediate approaches the substrate from predominantly the axial face of the cyclohexyl ring. The reason for the lack of complete stereoselection is not clear; however, it may be due to substrate motion in the binding site, arising from the binding of a nonoptimal substrate. While CVPAH does not appear to react with L-leucine or L-isoleucine, it gives fully uncoupled turnover with L-norleucine (R.T.C. and P.C.D.H., unpublished).

The chemical mechanism for the alkyl hydroxylation is likely to differ from the mechanism of aromatic hydroxylation, though the same oxygenating species (putatively the pterin 4a-hydroperoxide) is probably involved. The hydroperoxide may carry out the alkyl hydroxylation by one of two mechanisms, radical or direct oxygen insertion, neither of which will give rise to the observed cationic species deduced to be involved in the NIH shift (see above). Thus the same hydroxylating species may function by at least two different mechanisms. There are a number of chemical systems that will carry out saturated hydrocarbon hydroxylation in the absence of a metal. For example nitro-substituted perbenzoic acids (Schneider & Müller, 1985), perbenzoic acid (Fossey et al., 1985), ozone and ground state (triplet) oxygen (Zadok & Mazur, 1982), hydrogen peroxide/trifluoroacetic acid or trifluoroperacetic acid (Deno et al., 1977), dimethyldioxirane and derivatives (Murray et al., 1986; Mello et al., 1989), perfluorodialkyloxaziridines (Des-Marteau et al., 1993), and *tert*-butylhydroperoxide (Chenier et al., 1978) have all been used to perform this type of oxidation. Perbenzoic acid, triplet oxygen, and *tert*-butyl hydroperoxide all utilize the same mechanism, hydrogen radical abstraction at the site of reaction and then recombination of the carbon centered radical and a hydroxyl radical. The other species utilize a direct oxygen insertion into the C-H bond.

On the basis of the limited studies carried out to date, one cannot distinguish between either of the mechanisms mentioned above for PAH. If the 4a-hydroperoxy pterin is indeed the hydroxylating species, then it may behave like *tert*-butyl hydroperoxide and utilize a radical mechanism or like the nitro-substituted perbenzoic acids and utilize a direct oxygen insertion mechanism. An examination of the intrinsic isotope effect for the hydroxylation may be helpful in distinguishing these possibilities as the isotope effects for direct oxygen insertion are rather lower ($\approx$5) (Murray et al., 1985) than those for radical hydroxylation ($\approx$8). This problem may be addressed using either [2-^{2}H]- or [2,3,4,5,6-$^2H_5$]-cyclohexylalanine and examining the preference for replacement of ^{2}H or ^{1}H by OH. A study with various alternate substrates designed to trap any radical intermediates in the pathway may reveal further information on this problem.

A general feature of oxygenases requiring reducing cofactors is that under certain conditions the cofactor may be oxidized without any product formation. It was noted by Dix and Benkovic (1985) that using 4-chloro-L-phenylalanine as a substrate or unsubstituted tetrahydropterin as cofactor

partially uncoupled the PAH reaction. In their study roughly three pterins were oxidized for every substrate hydroxylated. Kaufman has observed 100% uncoupled pterin oxidation with lysolecithin activated RLPAH when using tyrosine as a substrate (Davis & Kaufman, 1988). Unlike RLPAH, however, CVPAH does not produce any coupled or uncoupled turnover with *p*-chlorophenylalanine or tyrosine. Tetrahydropterin is utilized as a cofactor by CVPAH; however, the reaction with phenylalanine is fully coupled. There is also no pterin oxidase activity of CVPAH in the presence of H_2O_2 or *m*-chloroperbenzoic acid which was also observed with the RLPAH (Dix & Benkovic, 1985).

In conclusion, all experiments on the mechanism of metal-independent hydroxylations by CVPAH produce results either identical with or equivalent to results obtained for the iron-dependent RLPAH. This suggests that the two hydroxylating intermediates are one and the same, and that the role of the non-heme iron in mammalian pterin dependent hydroxylases may be only tangential. Clearly, the iron of these hydroxylases is at the active site and required for activity, but no evidence exists that this iron transfers electrons or binds oxygen during the catalytic cycle. Now we have a precedent for accomplishing the exact same reactions with a metal free enzyme; therefore, there is no need to invoke an iron-containing intermediate in the reactions of iron-dependent aromatic amino acid hydroxylases. Experimental evidence, linking the non-heme iron to either electron transfer or oxygen binding during the catalytic cycle, should now be required before iron–oxo intermediates are further postulated as the hydroxylating agent for these enzymes. Such evidence will be difficult to produce as these intermediates are transient. At this point, the limited evidence favors the tetrahydropterin 4a-hydroperoxide as the common intermediate responsible for hydroxylations in both mammalian and bacterial systems. The observation of metal-independent stereoselective hydroxylation of a saturated carbon center is unprecedented. That this difficult reaction can be carried out using only a pterin cofactor requires a reassessment of the oxygenating power of the proposed 4a-hydroperoxy pterin. The problem of oxygen binding in the metal-free system remains to be solved.

REFERENCES

Abita, J.-P., Parniak, M., & Kaufman, S. (1986) *J. Biol. Chem. 259*, 14560–14566.

Beaty, N. B., & Ballou, D. P. (1980) *J. Am. Chem. Soc. 255*, 3817–3822.

Blackburn, N. J., Strange, R. W., Carr, R. T., & Benkovic, S. J. (1992) *Biochemistry 31*, 5298–5303.

Brundish, A., & Wade, D. B. (1976) *J. Chem. Soc. Perkin Trans. I*, 2186–2189.

Bumpus, J. (1974) *J. Med. Chem. 17*, 1156–1163.

Carr, R. T., & Benkovic, S. J. (1993) *Biochemistry 32*, 14132–14138.

Chenier, J. H. B., Tong, S. B., & Howard, A. J. (1978) *Can. J. Chem. 56*, 3047–3054.

Curti, B., Ronchi, S., & Zanetti, G. (1991) in *Flavins and Flavoproteins 1990* (Ayling, J. E., Ed.) pp 247–250, Walter de Gruyter & Co., Berlin.

Daley, J., & Guroff, G. (1968) *Arch. Biochem. Biophys. 125*, 136–141.

Davis, M. D., & Kaufman, S. (1988) *J. Biol. Chem. 263*, 17312–17316.

Davis, M. D., & Kaufman, S. (1993) *Arch. Biochem. Biophys. 304*, 9–16.

Deno, N. C., Jedziniak, E. J., Messer, L. A., Meyer, M. D., Stroud, S. G., & Tomesko, E. S. (1977) *J. Org. Chem. 33*, 2503–2510.

DesMarteau, D. D., Donadelli, A., Montanari, V., Petrov, V. A., & Resnati, G. (1993) *J. Am. Chem. Soc. 115*, 4897–4899.

Dix, T. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 5839–5845.

Dix, T. A., & Benkovic, S. J. (1988) *Acc. Chem. Res. 21*, 101–107.

Eberlein, G., Bruice, T. C., Lazarus, R. H., & Benkovic, S. J. (1984) *J. Am. Chem. Soc. 106*, 7916–7924.

Fisher, D. B., Kirkwood, R., & Kaufman, S. (1972) *J. Biol. Chem. 247*, 5161–5167.

Fitzpatrick, P. F. (1991) *Biochemistry 30*, 6386–6392.

Fossey, J., Lefort, D., Massoudi, M., Nedelec, J.-Y., & Sorba, J. (1985) *Can. J. Chem. 63*, 678–681.

Gibbs, B. S., Wojchowski, D., & Benkovic, S. J. (1993) *J. Biol. Chem. 268*, 8046–8052.

Gold, V. (1964) in *Advances in Physical Organic Chemistry* (Gold, V., Ed.) Vol. 2, pp 164–187, Academic Press, New York.

Gottschall, D. W., Dietrich, R. F., Benkovic, S. J., & Shiman, R. (1982) *J. Biol. Chem. 257*, 845–851.

Grovenstein, E., & Aprahamian, N. S. (1962) *J. Am. Chem. Soc. 84*, 212–219.

Guengerich, F. P. (1990) in *Biological Oxidation Systems* (Reddy, C., Hamilton, G. A., & Madyastha, K. M., Eds.) Vol. 1, pp 51–67, Academic Press, New York.

Guilbault, G. G., & Kramer, D. M. (1964) *Anal. Chem. 36*, 2495–2500.

Guroff, G., & Rhoads, C. A. (1967) *J. Biol. Chem. 242*, 3641–3645.

Guroff, G., Reifsnyder, C. A., & Daley, J. (1966a) *Biochem. Biophys. Res. Commun. 24*, 720–724.

Guroff, G., Levitt, M., Daley, J., & Udenfriend, S. (1966b) *Biochem. Biophys. Res. Commun. 25*, 253–259.

Guroff, G., Daley, J., Jerina, D. M., Renson, J., Witkop, B., & Udenfriend, S. (1967) *Science 157*, 1524–1530.

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

Kaufman, S., & Mason, K. (1983) *J. Biol. Chem. 257*, 14667–14678.

Mager, H. I. X., Addiuk, R., & Berends, W. (1967) *Recl. Trav. Chim. Pays-Bas 86*, 833–851.

Massey, V., & Hemmerich, P. (1976) *Enzymes (3rd. ed.) 12*, 191–199.

Mello, R., Fiorentino, M., Fusco, C., & Curci, R. (1989) *J. Am. Chem. Soc. 111*, 6749–6754.

Miller, M. R., McClure, D., & Shiman, R. (1975) *J. Biol. Chem. 250*, 1132–1138.

Murray, R. W., Jeyaraman, R., & Mohan, L. (1986) *J. Am. Chem. Soc. 108*, 2470–2474.

Onishi, A., Liotta, L. J., & Benkovic, S. J. (1991) *J. Biol. Chem. 266*, 18454–18459.

Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1986) *Biochemistry 25*, 6611–6619.

Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1987) *Methods Enzymol. 142*, 50–56.

Pember, S. O., Johnson, K. A., Villafranca, J. J., & Benkovic, S. J. (1989) *Biochemistry 28*, 2124–2133.

Pretsch, E., Clerc, T., Seibl, J., & Simon, W. (1989) *Spectral Data for Structure Determination of Organic Compounds*, Springer-Verlag, Berlin.

Schneider, H.-J., & Müller, W. (1985) *J. Org. Chem. 50*, 4609–4614.

Shiman, R. (1985) in *Folates and Pterins* (Blakely, R. L., & Benkovic, S. J., Eds.) pp 179–185, Wiley-Interscience, New York.

Siegmund, H., & Kaufman, S. (1991) *J. Biol. Chem. 266*, 2903–2910.

White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–336.

Zadok, E., & Mazur, Y. (1982) *Angew. Chem., Int. Ed. Engl. 21*, 303–308.