Regulation of Oxidation–Reduction Potentials of Anthranilate Hydroxylase from *Trichosporon cutaneum* by Substrate and Effector Binding†

Gunnlaug H. Einarsdottir,‡ Marian T. Stankovich,*,‡ Justin Powlowski,§ David P. Ballou,§ and Vincent Massey§

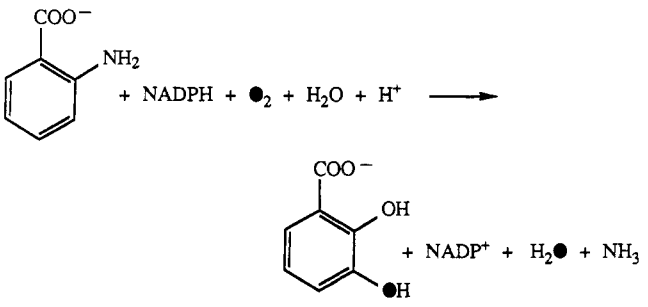
Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455, and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

Received September 26, 1988; Revised Manuscript Received January 23, 1989

ABSTRACT: The pH dependence of the redox behavior of anthranilate hydroxylase from *Trichosporon cutaneum* in its uncomplexed and anthranilate-complexed forms, as well as the effects on the reduction potential, at pH 7.4, of enzyme in complex with 3-methylanthranilate, salicylate, 3-acetylpyridine adenine dinucleotide phosphates, and azide plus anthranilate, is described. At pH 7.4 the midpoint potential of uncomplexed enzyme ($EFl_{ox}/EFl_{red}H^-$) is −0.229 V vs SHE, close to that of free flavin. The aromatic substrates and effector all shift the midpoint potential value in a positive direction by 0.068–0.100 V. This shift results in thermodynamically more favorable reduction of the substrate/effector-complexed enzyme by NADPH. Consistent with thermodynamic considerations, the aromatic substrates (or effector) are bound to the reduced enzyme 2–4 orders of magnitude more tightly than to the oxidized enzyme. The tighter binding of the substrate to the two-electron-reduced enzyme may be related to the double hydroxylation reaction performed by this enzyme, which is a more complex reaction than is carried out by typical flavoprotein hydroxylases. The acetylpyridine nucleotides appear to have no significant regulatory role.

Flavoprotein hydroxylases that activate molecular oxygen for insertion into the benzene ring are elaborated by microorganisms that can grow using aromatic compounds as the sole source of carbon (Dagley, 1982). These enzymes are usually quite specifically induced in response to substrates that require an additional hydroxyl group before further metabolism can occur. A number of different hydroxylases have been isolated from bacterial sources, and a smaller number have been obtained from eukaryotic organisms (Dagley, 1982).

Recently, the isolation of anthranilate hydroxylase (EC 1.14.12.2) from the eukaryote *Trichosporon cutaneum* was reported (Powlowski et al., 1987a). This enzyme catalyzes a unique hydrolytic deamination at the 2-position of the substrate in addition to hydroxylation using O_2 at the 3-position:



Nevertheless, the oxidative reactions of the flavin prosthetic group are very similar to those observed with other flavin-containing monooxygenases (Ballou, 1982; Powlowski et al., 1985). Several other properties are shared with enzymes in this class. For example, the enzyme-bound aromatic substrate or effector enhances the rate of enzyme reduction by NADPH by several orders of magnitude over the rate observed with the ligand-free enzyme (Powlowski et al., 1985, 1987a; Massey et al., 1982). Also, monovalent anions stabilize intermediates in the oxidative half-reaction. In contrast to other flavoprotein hydroxylases, anthranilate hydroxylase exhibits a slow initial turnover, followed by more rapid reactions in succeeding turnovers. This phenomenon appears to be due to slow conformational changes, related to substrate binding and enzyme reduction in the initial turnover, that result in an "activated" form of the enzyme for subsequent turnovers (Powlowski et al., 1987b).

The redox properties of two flavoprotein monooxygenases from prokaryotes have been determined, i.e., *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (Williamson et al. 1988) and salicylate hydroxylase from *Pseudomonas cepacia* (Einarsdottir et al., 1988). The more complex reaction catalyzed by anthranilate hydroxylase, and the observation of conformational changes upon substrate binding and during the initial turnover, suggested that the redox chemistry of this enzyme might be more regulated by substrate binding than was observed with the other two hydroxylases. To further clarify the role of thermodynamics in the enzyme mechanism, we measured the oxidation–reduction potentials of the uncomplexed enzyme and of the enzyme in complex with substrate, effector, and monovalent anion (inhibitor). The effect of NADPH analogues was also addressed. In this paper the redox properties of anthranilate hydroxylase are compared to those of salicylate hydroxylase and *p*-hydroxybenzoate hydroxylase.

Materials and Methods

Materials. Anthranilate hydroxylase was isolated from *Trichosporon cutaneum* according to the method previously published (Powlowski et al., 1987a). Glass-distilled water was used in all experiments. Anthranilic acid from Aldrich (Milwaukee, WI) was recrystallized from benzene or water. 3-Methylanthranilic acid from Aldrich was recrystallized from ethanol. Sodium salicylate from EM Science (Gibbstown, NJ) was recrystallized from water. Methyl viologen and pheno-

† This work was supported by Grants GM 29344 (to M.T.S.), GM 20877 (to D.P.B.), and GM 11106 (to V.M.). G.H.E. is the recipient of a Shell predoctoral fellowship.

* To whom correspondence should be addressed.
‡ University of Minnesota.
§ University of Michigan.

0006-2960/89/0428-4161$01.50/0

Table I: Redox Potentials for Uncomplexed Anthranilate Hydroxylase in 0.050 M Potassium Phosphate Buffer and 0.1 mM EDTA at 4 °C

pH	indicator dye	E_m of indicator (V)	concn indicator (μM)	concn enzyme (μM)	E_m of enzyme (V)	slope (V)	no. of points
6.5	LFA	−0.197	1.8	16.2	−0.204 (R)[a]	0.026	7
7.0	LFA	−0.212	4.0	20.7	−0.220 (R)	0.023	5
7.0	LFA	−0.212	4.0	20.7	−0.216 (O)	0.025	5
7.4	LFA	−0.227	2.1	14.6	−0.228 (R)	0.030	6
7.4	LFA	−0.227	2.1	14.6	−0.223 (O)	0.024	7
7.4	LFA	−0.227	2.6	13.1	−0.229 (R)	0.031	7
7.7	LFA	−0.242	2.4	16.0	−0.240 (R)	0.023	5
7.7	LFA	−0.242	2.4	16.0	−0.238 (O)	0.026	6
8.0	LFA	−0.248	1.8	14.2	−0.254 (R)	0.027	6
8.0	PHE	−0.255	1.8	13.0	−0.250 (R)	0.024	6

[a] (R) and (O) indicate potentiometric titrations performed in the reductive (R) and oxidative (O) directions.

safranine were purchased from British Drug Houses (Poole, England). Lumiflavin 3-acetate was the generous gift of Dr. S. Ghisla, University of Konstanz, FRG. Riboflavin and 2-hydroxy-1,4-naphthoquinone were purchased from Eastman Kodak (Rochester, NY). 8-Chlororiboflavin was the generous gift of Dr. J. P. Lambooy, University of Maryland. Pyocyanine was synthesized by photochemical decomposition of phenazine methosulfate (McIlwain, 1937). 3-Acetylpyridine adenine dinucleotide phosphate was purchased from Sigma (St. Louis, MO). The reduced form of 3-acetylpyridine adenine dinucleotide was synthesized enzymatically (Ryerson et al., 1982). The final product was purified by ion-exchange chromatography, and the fractions containing product were concentrated with a YC05 membrane (Amicon). Finally, samples were desalted on a CP-2 (Bio-Rad) column. The purity of the final product was verified by FPLC (Orr & Blanchard, 1984).

Methods. Coulometric titrations were performed as previously described (Stankovich, 1980; Stankovich & Fox, 1983). Estimation of anthranilate hydroxylase concentration was based on the molar absorptivity of 11 630 M^{-1} cm^{-1}/flavin, determined for the uncomplexed enzyme and with an appropriate correction for ligand-induced perturbation. Enzyme concentrations refer to enzyme-bound FAD[1] throughout this paper. All experiments were performed in 0.05 M potassium phosphate buffer–0.1 mM EDTA at 4 °C unless otherwise stated. All electrochemical experiments contained 50 μM methyl viologen as a mediator to facilitate reduction. The appearance of the spectrum of reduced methyl viologen indicates that a titration is complete.

Potentiometric titrations were performed as previously described (Stankovich, 1980; Stankovich & Fox, 1983). In control experiments all redox indicator dyes were titrated individually to obtain the redox potentials and spectral characteristics. This made it possible to correct spectra for the presence of indicator dyes. Potential values were measured at one pH with two structurally different redox indicators to ensure the measured potentials of the enzyme were not dependent on the indicator. Potentiometric titrations performed in the oxidative direction contained ~10 μM pyocyanine to mediate electron transfer between the electrode and the enzyme. All potential values are reported versus the standard hydrogen electrode (SHE). All potential values for potentiometric titrations in the reductive direction refer to the second reduction of the enzyme. In the first reduction, the system required 3 h per point to reach equilibrium compared to 1.5–2 h per point for titrations in the oxidative or the second reductive direction to reach the same equilibrium.

From the shift in midpoint potential upon ligand binding and the known dissociation constant of the ligand from the oxidized enzyme K_{ox}, the dissociation constant for the reduced enzyme K_{red} can be calculated. At 4 °C

$$E_{m,ligand} = E_{m,free} + \frac{0.055}{n} \log \frac{1 + [ligand]/K_{red}}{1 + [ligand]/K_{ox}}$$

where $E_{m,ligand}$ is the midpoint potential value obtained at specific ligand concentration ([ligand]) and $E_{m,free}$ is the midpoint potential value for the free, uncomplexed enzyme.

Ligand binding was determined by titrating the enzyme with aliquots of the ligand and measuring the resulting perturbations of the enzyme absorbance spectrum. Data were analyzed either by a double-reciprocal method (Benesi and Hildebrand, 1949) or by a computerized, weighted nonlinear regression method (Duggleby, 1981). The equation used was

$$\frac{\Delta A}{\Delta A_{max}} = \frac{(L_T + E_T + K_d) - \sqrt{(L_T + E_T + K_d)^2 - 4E_T L_T}}{2E_T}$$

where E_T is the total enzyme concentration, L_T is the total ligand concentration, and ΔA_{max} and K_d are the fitted variables.

Results

Titrations of Uncomplexed Anthranilate Hydroxylase. Spectra obtained during coulometric titration of uncomplexed anthranilate hydroxylase at pH 8.0 are shown in Figure 1A. Formation of an isosbestic point at 342 nm suggests that there are only two enzyme species present during the titration. Molar absorptivity for the two-electron-reduced enzyme calculated from this experiment is 1200 M^{-1} cm^{-1} at 456 nm. No spectral evidence for semiquinone formation is observed during titrations over the pH range 6.5–8.0. Methyl viologen, a positively charged one-electron donor, is capable of transferring electrons to the enzyme faster than dithionite, a negatively charged one-electron donor. Thus, with methyl viologen, electron transfer was complete upon addition of each increment of charge, while with dithionite it took about 1 h per increment, indicating that positively charged methyl viologen has better access to the flavin site than negatively charged dithionite.

The pH dependence of the redox potential was determined over the pH range 6.5–8.0. Results for the midpoint potential values for the uncomplexed enzyme are shown in Table I. At pH 7.4, a midpoint potential value E_m of −0.228 V was ob-

[1] Abbreviations: $AcPyADP^+$, 3-acetylpyridine adenine dinucleotide phosphate; AcPyADPH, 3-acetylpyridine adenine dinucleotide phosphate, reduced form; Ant, anthranilate; 8ClRf, 8-chlororiboflavin; EDTA, ethylenediaminetetraacetic acid; EFl_{ox}, oxidized anthranilate hydroxylase; $EFl_{red}H^-$, two-electron-reduced anthranilate hydroxylase; EFl_{ox}·Ant, anthranilate-bound oxidized anthranilate hydroxylase; $EFl_{red}H^-$·Ant, two-electron-reduced anthranilate-bound anthranilate hydroxylase; FAD, flavin adenine dinucleotide; 2HNQ, 2-hydroxy-1,4-naphthoquinone; IDS, indigodisulfonate; LFA, lumiflavin 3-acetate; PHE, phenosafranine; Rf, riboflavin.

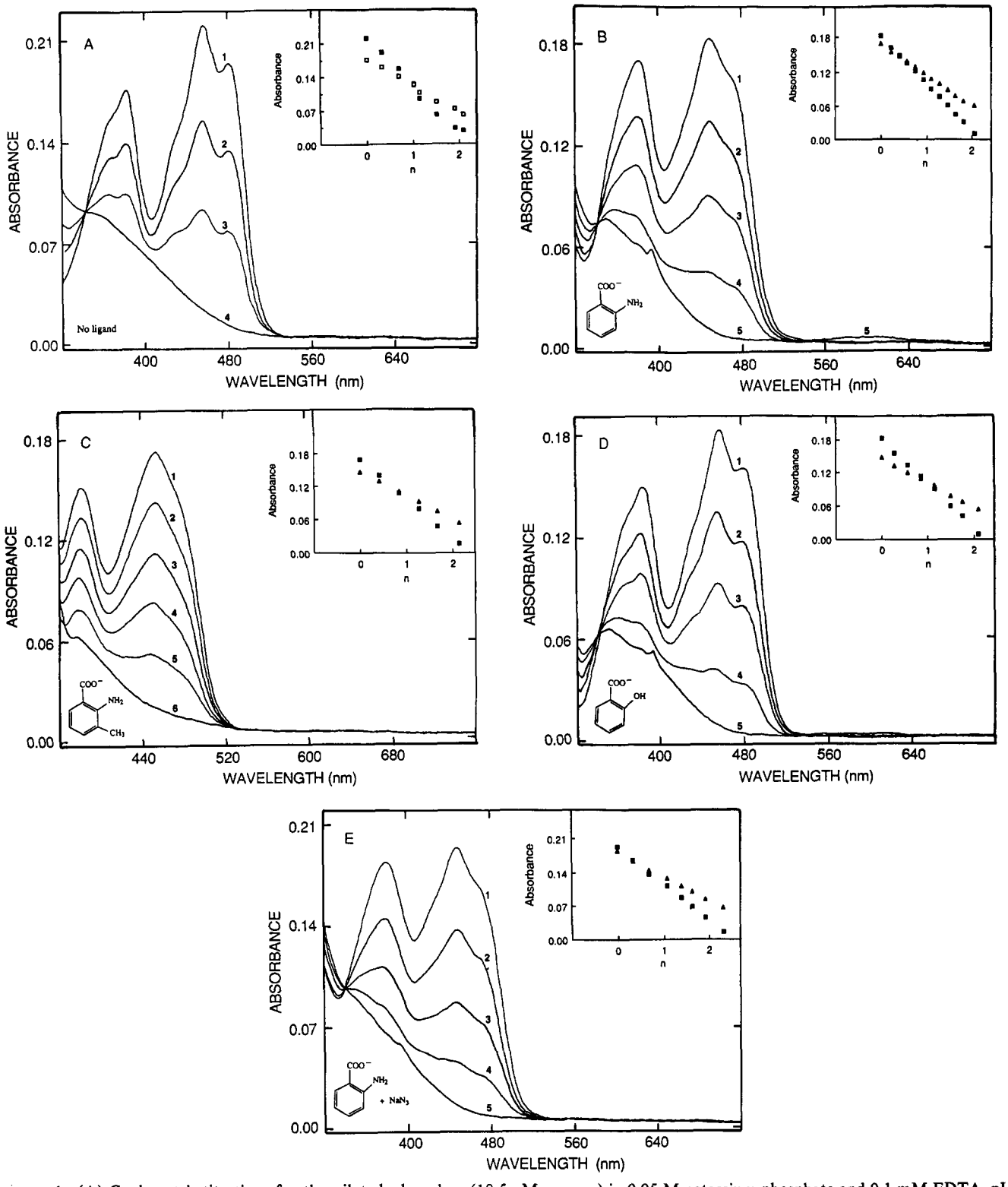


FIGURE 1: (A) Coulometric titration of anthranilate hydroxylase (18.5 μM enzyme) in 0.05 M potassium phosphate and 0.1 mM EDTA, pH 8.0, at 4 °C: (1) oxidized enzyme; (2) n = 0.69; (3) n = 1.5; (4) reduced enzyme. The inset shows the absorbance vs number of reducing equivalents added plotted for (■) 456 and (□) 383 nm. (B) Coulometric titration of anthranilate hydroxylase in the presence of anthranilate (15.1 μM enzyme) in 0.05 M potassium phosphate, 0.1 mM EDTA, and 0.6 mM anthranilate, pH 7.6, at 4 °C: (1) oxidized enzyme; (2) n = 0.57; (3) n = 1.11; (4) n = 1.64; (5) reduced enzyme, spectrum of reduced methyl viologen appears (λ_{max}, 603 and 395 nm). The inset shows the absorbance vs number of reducing equivalents added plotted for (■) 450 and (▲) 381 nm. (C) Coulometric titration of anthranilate hydroxylase in the presence of 3-methylanthranilate (14.2 μM enzyme) in 0.05 M potassium phosphate, 0.1 mM EDTA, and 0.02 M 3-methylanthranilate, pH 7.6, at 4 °C: (1) oxidized enzyme; (2) n = 0.42; (3) n = 0.83; (4) n = 1.27; (5) n = 1.66; (6) reduced enzyme. The inset shows the absorbance vs number of reducing equivalents added plotted for (■) 456 and (▲) 382 nm. (D) Coulometric titration of anthranilate hydroxylase in the presence of salicylate (15.1 μM enzyme) in 0.05 M potassium phosphate, 0.1 mM EDTA, and 1.2 mM sodium salicylate, pH 7.6, at 4 °C: (1) oxidized enzyme; (2) n = 0.57; (3) n = 1.15; (4) n = 1.74; (5) reduced enzyme, spectrum of reduced methyl viologen appears (λ_{max}, 603 and 395 nm). The inset shows the absorbance vs number of reducing equivalents added plotted for (■) 456 and (▲) 384 nm. (E) Coulometric titration of anthranilate hydroxylase in the presence of azide and anthranilate (16.5 μM enzyme) in 0.05 M potassium phosphate, 0.1 mM EDTA, 0.1 M sodium azide, and 0.6 mM anthranilate, pH 7.6, at 4 °C: (1) oxidized enzyme; (2) n = 0.69; (3) n = 1.39; (4) n = 1.93; (5) reduced enzyme. The inset shows the absorbance vs number of reducing equivalents added plotted for (■) 449 and (▲) 380 nm.

Table II: Redox Potentials for Ligand-Complexed Anthranilate Hydroxylase in 0.050 M Potassium Phosphate Buffer and 0.1 mM EDTA at 4 °C

pH	indicator dye	E_m of indicator (V)	concn		E_m of enzyme (V)	slope (V)	no. of points
			indicator (μM)	enzyme (μM)			
		Anthranilate Hydroxylase in the Presence of Anthranilate (0.6 mM)					
6.5	8ClRf	−0.126	2.5	13.1	−0.111 (R)[a]	0.034	7
7.0	8ClRf	−0.143	3.1	14.9	−0.125 (R)	0.027	7
7.0	8ClRf	−0.143	3.1	14.9	−0.118 (O)	0.021	7
7.4	8ClRf	−0.153	3.2	17.8	−0.137 (R)	0.032	5
7.4	8ClRf	−0.153	3.2	17.8	−0.132 (O)	0.026	4
7.4	8ClRf	−0.153	2.5	15.2	−0.141 (R)	0.031	7
7.4	8ClRf	−0.153	2.5	15.2	−0.131 (O)	0.023	6
7.4	2HNQ	−0.148	3.7	13.5	−0.144 (R)	0.030	6
7.9	8ClRf	−0.165	2.5	13.6	−0.150 (R)	0.032	7
7.9	8ClRf	−0.165	2.6	11.2	−0.154 (R)	0.033	5
		Anthranilate Hydroxylase in the Presence of 3-Methylanthranilate (0.02 M)					
7.4	Rf	−0.199	1.8	14.1	−0.161 (R)	0.031	6
7.4	8ClRf	−0.149	2.4	15.5	−0.160 (R)	0.028	7
7.4	8ClRf	−0.149	2.4	15.5	−0.154 (O)	0.026	7
		Anthranilate Hydroxylase in the Presence of Salicylate (1.2 mM)					
7.4	8ClRf	−0.150	1.7	14.7	−0.128 (R)	0.026	6
7.4	8ClRf	−0.150	2.1	14.7	−0.128 (R)	0.030	5
7.4	8ClRf	−0.150	2.1	14.7	−0.122 (O)	0.030	5
		Anthranilate Hydroxylase in the Presence of Azide (0.1 M) and Anthranilate (0.6 mM)					
7.5	Rf	−0.196	2.3	17.3	−0.175 (R)	0.028	7
7.4	Rf	−0.196	2.5	15.4	−0.176 (R)	0.029	5
7.4	Rf	−0.196	2.5	15.4	−0.169 (O)	0.026	5

[a] (R) and (O) indicate potentiometric titrations performed in the reductive (R) and oxidative (O) directions.

tained in the reductive direction, and an E_m of −0.223 V was calculated in the oxidative direction. Plots of measured potential versus logarithm ($EFl_{ox}/EFl_{red}H^-$) gave slopes of 0.030 and 0.024 V in the reductive and oxidative directions, respectively, both of which are close to the Nernst value of 0.027 V for a two-electron transfer at 4 °C. This clearly shows the reversibility of the electron transfer. A separate reductive potentiometric titration gave a midpoint potential of −0.229 V with a 0.031-V Nernst slope. The reversibility of electron transfer was also tested at pH 7.0 and 7.7. The difference between midpoint potential values obtained in reductive and oxidative directions at all three pH values agrees within 0.002 and 0.005 V.

Binding of the indicator dyes LFA and PHE can be ruled out since excellent agreement on the midpoint potential values, within 0.004 V, was obtained with these two structurally different indicator dyes (see Table I).

The midpoint potential behavior for anthranilate hydroxylase over the pH range 6.5–8.0 is shown in Figure 2, line A. There is a 0.032-V potential/pH unit change for the midpoint potential in the reductive direction, which is consistent with a two-electron, one-proton transfer. At pH 7.4

$$EFl_{ox} + 2e^- + H^+ \rightleftharpoons EFl_{red}H^-$$
$$E_m = -0.229 \text{ V vs SHE}$$

Titrations of Anthranilate-Bound Anthranilate Hydroxylase. Electrochemical reduction of anthranilate hydroxylase in the presence of 0.6 mM anthranilate at pH 7.4 is shown in Figure 1B. The spectrum of the anthranilate-bound oxidized enzyme is quite different from that of the ligand-free enzyme; the λ_{max} is shifted to 450 nm, and the flavin peaks are less resolved. A molar absorptivity of 600 M^{-1} cm^{-1} was calculated for the reduced enzyme–substrate complex at 450 nm. An isosbestic point formed at 342 nm for oxidized and reduced enzyme. There are no spectrally detectable intermediates in going from the oxidized to the two-electron-reduced enzyme over the pH range 6.5–7.9. In contrast to the ligand-free enzyme, reduction of anthranilate hydroxylase with dithionite in the presence of anthranilate was as rapid as the electrochemical reduction.

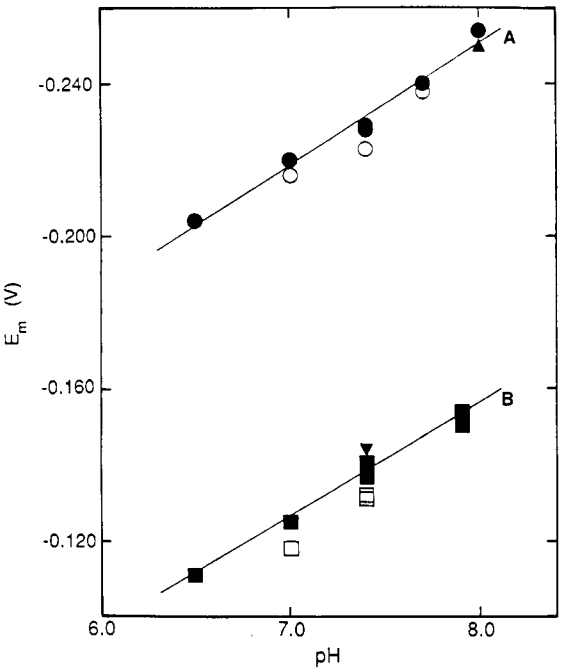


FIGURE 2: Potential vs pH behavior of anthranilate hydroxylase in 0.05 M potassium phosphate and 0.1 mM EDTA at 4 °C. Line A represents the uncomplexed enzyme and line B the enzyme in presence of 0.6 mM anthranilate. Redox indicator dyes used were (●) LFA (reductive direction), (○) LFA (oxidative direction), (▲) PHE (reductive direction), (■) 8ClRf (reductive direction), (□) 8ClRf (oxidative direction), and (▼) 2HNQ (reductive direction).

The electrochemical potential of anthranilate hydroxylase complexed with anthranilate was determined over the pH range 6.5–7.9. Results for the midpoint potential values for the anthranilate-bound enzyme and indicator dyes used are summarized in Table II. The potential/pH behavior for the anthranilate-bound enzyme is shown in Figure 2, line B. There is a 0.030-V potential/pH change for midpoint potentials in

the reductive direction, which is consistent with a two-electron, one-proton transfer as was found for the uncomplexed enzyme.

The reversibility of the electron transfer was tested at pH 7.0 and 7.4. Differences in midpoint potential values obtained in titrations performed in the reductive and oxidative directions are between 0.005 and 0.01 V, slightly greater than was seen for the uncomplexed enzyme. A certain amount of hysteresis is expected due to the approach to equilibrium from opposite directions. However, this hysteresis is larger than that seen for other hydroxylases (Williamson et al., 1988; Einarsdottir et al.; 1988).

A reductive potentiometric titration of anthranilate-bound anthranilate hydroxylase at pH 7.4 using 2HNQ as the redox indicator dye gave an E_m of −0.144 V with a Nernst slope of 0.030 V. Two separate reductive potentiometric titrations under the same conditions using 8ClRf as redox indicator dye gave E_m values of −0.141 and −0.137 V with Nernst slopes of 0.031 and 0.032 V, respectively. These three E_m values are in good agreement and give an average value of −0.141 V as the midpoint potential in the reductive direction for anthranilate hydroxylase at pH 7.4:

$$EFl_{ox}\cdot Ant + 2e^- + H^+ \rightleftharpoons EFl_{red}H^-\cdot Ant$$
$$E_m = -0.141 \text{ V vs SHE}$$

This corresponds to a 0.088-V positive shift in the reductive midpoint potential value upon anthranilate binding. By use of this potential shift and a value of 30 μM for K_{ox},[2] the dissociation constant of anthranilate from the two-electron reduced enzyme, K_{red}, is calculated to be 18 nM. Consistent with this is the observation that titrations of reduced enzyme with anthranilate showed approximately 1:1 saturation of the spectral change with ligand addition, indicating very tight binding (data not shown). Thus, anthranilate remains tightly bound through the reductive cycle of the reaction and is more tightly bound to the reduced than to the oxidized enzyme by a factor of 1700.

Effect of 3-Methylanthranilate. 3-Methylanthranilate is also a substrate for anthranilate hydroxylase, although the product of the reaction is unstable and has not yet been isolated or identified (Powlowski et al., 1987a). Figure 1C shows the coulometric titration of the enzyme at pH 7.4 in the presence of 0.02 M 3-methylanthranilate. The spectrum of the oxidized enzyme has lost all resolution for both flavin peaks in the presence of this substrate. Again, no spectrally detectable intermediates were observed upon reduction of the enzyme. The isosbestic point for the enzyme, which occurs at 340 nm for free enzyme or enzyme bound to other substrates, is obscured in the presence of 3-methylanthranilate. 3-Methylanthranilate has a very high molar absorptivity at wavelengths below 360 nm, and it was present at high concentration in both the enzyme solution and the reference cuvette. As a result very little light was transmitted to the photomultiplier tube; therefore, the signal obtained in this region is meaningless.

The effect of 3-methylanthranilate on the midpoint potential was measured at pH 7.4. With Rf and 8ClRf as indicator dyes, midpoint potential values of −0.161 and −0.160 V were obtained in the reductive direction with Nernst slopes of 0.031 and 0.028 V, respectively. In the potentiometric titration with 8ClRf, a midpoint potential value of −0.154 V was obtained in the oxidative direction with a 0.026-V Nernst slope. This hysteresis between potential values is comparable to that obtained in reductive and oxidative directions with anthranilate-bound enzyme. 3-Methylanthranilate shifts the midpoint potential value less than does anthranilate, the native substrate.

[2] J. Powlowski, unpublished results.

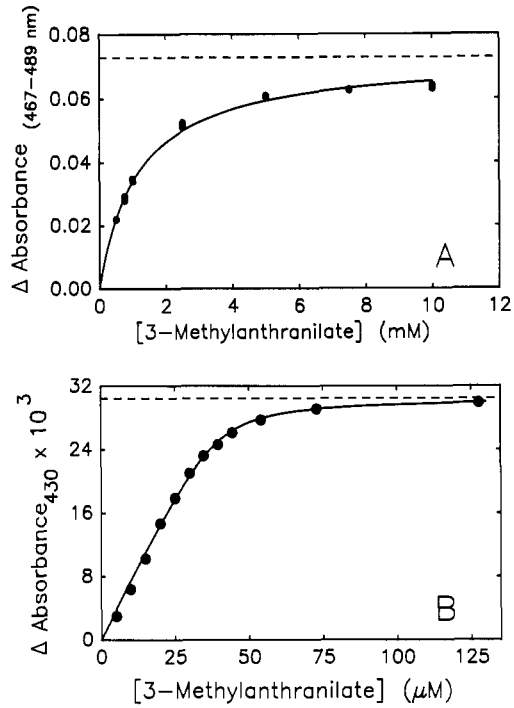


FIGURE 3: Titration of anthranilate hydroxylase with 3-methylanthranilate at 3 °C as monitored by spectral perturbations. (A) Titration of oxidized enzyme (10.6 μM FAD) with 3-methylanthranilate. This experiment was performed by mixing enzyme with ligand in a scanning stopped-flow spectrometer and recording the final spectra after the slow absorbance change ceased. Difference spectra were calculated for two separate shots. Filled circles (●) are the data points, and the solid line (—) is the calculated (Duggleby, 1981) best fit with K_d = 1.13 mM. ΔA_{max} = 0.73 (– –). (B) Titration of reduced enzyme (39 μM) with 3-methylanthranilate. The enzyme, under an atmosphere of nitrogen, was reduced with an excess of sodium dithionite, and then aliquots of a solution of 3-methylanthranilate were added anaerobically. Spectra were recorded vs buffer. The data points (●) are plotted along with the calculated best fit (—), K_d = 1.65 μM; the dashed line is the calculated ΔA_{max}. The first two data points were not used in the fit.

Binding of 3-methylanthranilate to both oxidized and reduced enzyme was measured from spectral perturbations upon addition of ligand, K_{ox} = 1.1 mM and K_{red} = 1.7 μM (see Figure 3). On the basis of these K_d values, a positive potential shift of 0.077 V would be expected. This is in good agreement with data presented here, where a potential shift of +0.068 V was measured. A dissociation constant for 3-methylanthranilate from the reduced enzyme, K_{red}, calculated from the measured reductive potential shift and K_{ox}, is 3.5 μM.

Effects of Salicylate. Salicylate is an effector for anthranilate hydroxylase and is not hydroxylated in the enzyme reaction. After salicylate is bound to the enzyme, the FAD moiety is reduced rapidly by NADPH. The reduced species reacts with O_2 to form the flavin 4a-hydroperoxide, which then decays to oxidized flavin and H_2O_2 without hydroxylation of salicylate (Powlowski et al., 1988). Binding of salicylate to the oxidized form of the enzyme does not appear to be a simple process, and it is further complicated by the fact that the observed spectral changes are very small (see Figure 1D). Spectral changes observed for binding to the oxidized form of the enzyme are also qualitatively somewhat different from those observed with anthranilate. These results are discussed more fully in a separate paper (Powlowski et al., 1988). As an approximation, salicylate appears to bind to the oxidized form of the enzyme 5–8 times less tightly than does anthranilate. As was observed with anthranilate, the K_d for the dissociation of salicylate from the reduced form of the enzyme is too low to measure accurately.

The electrochemical reduction of anthranilate hydroxylase in the presence of 1.2 mM sodium salicylate at pH 7.4 is shown in Figure 1D. The spectrum for the reduced salicylate-bound anthranilate hydroxylase differs significantly from that of the ligand-free reduced enzyme, and the molar absorptivity for the salicylate-bound two-electron-reduced enzyme is 600 M^{-1} cm^{-1} at 456 nm. Upon addition of reducing charge, the enzyme goes directly from the oxidized enzyme to the two-electron-reduced enzyme with an isosbestic point forming at 343 nm and with no spectrally detectable formation of semiquinone.

Salicylate had the greatest effect on the midpoint potential value. At 1.2 mM sodium salicylate concentration and pH 7.4, a midpoint potential of −0.128 V was measured in the reductive direction in two experiments, with Nernst slopes of 0.030 and 0.026 V. This corresponds to a 0.101-V positive shift in reductive midpoint potential value. The calculated dissociation constant for salicylate binding to two-electron-reduced enzyme is in the range of 28–42 nM, based on the shift in the reductive midpoint potential value and the approximation of a K_{ox} of 150–240 μM. This reflects a very tight binding to the two-electron-reduced enzyme as was observed for anthranilate binding to reduced enzyme.

Effects of Azide and Anthranilate. Azide binds to anthranilate hydroxylase, causing stabilization of the 4a-hydroxyflavin intermediate in the oxidative half-reaction.[2] This phenomenon has been observed with all flavoprotein aromatic ring hydroxylases for which the oxidative half-reactions have been examined [see Ballou (1982) for a review]. Titration of the oxidized, ligand-free enzyme with sodium azide indicated a prohibitively high K_{ox}, complicated spectral changes, and significant dissociation of flavin at a high concentration of azide. By contrast, binding of azide to oxidized enzyme in the presence of 0.6 mM anthranilate proceeded isosbestically until 70–140 mM sodium azide had been added. Furthermore, the observed spectral changes were not those expected for displacement of anthranilate from the enzyme upon azide binding, and additional anthranilate (up to 1.2 mM) caused no significant reversal of the changes. At concentrations of 0.1 M sodium azide and 0.6 mM anthranilate, 98% of the flavin chromophore was retained upon filtration in a Centricon-30 apparatus, indicating that the observed changes were not due to flavin release under these conditions. Thus it appears that azide binds to the oxidized enzyme–anthranilate complex, and a K_{ox} of 9.8 mM was calculated.

The coulometric titration of anthranilate hydroxylase in the presence of 0.1 M azide and 0.6 mM anthranilate, pH 7.4, is shown in Figure 1E. Upon addition of charge the oxidized enzyme goes directly to the two-electron-reduced form with no spectral evidence for semiquinone formation. An isosbestic point is observed at 340 nm in the titration. The two-electron-reduced enzyme in the presence of azide and anthranilate has a molar absorptivity of 700 M^{-1} cm^{-1} at 450 nm.

The effect on the midpoint potential was measured at pH 7.4 (see Table II). Midpoint values of −0.175 and −0.176 V were obtained in the reductive direction with Nernst slopes of 0.028 and 0.029 V, respectively. An oxidative potentiometric titration gave an E_m of −0.169 V with a 0.026-V Nernst slope; so again a small hysteresis is seen when equilibrium is reached from both sides. The midpoint potential value lies between that of the uncomplexed enzyme and that of the anthranilate-bound enzyme. The potential shift to a more negative value than that of the anthranilate-bound enzyme suggests that the azide is more tightly bound to the oxidized anthranilate–complexed enzyme. This is in contrast to the behavior of the aromatic compounds studied here, which tend to bind tighter to the two-electron-reduced form of the enzyme.

Effects of 3-Acetylpyridine Adenine Dinucleotide Phosphate. The $AcPyADP^+$/AcPyADPH redox couple has a midpoint potential [−0.260 V at pH 7.0 (Massey & Palmer, 1962)] similar to that of the uncomplexed enzyme (−0.220 V at pH 7.0). Its effect on the midpoint potential value of the uncomplexed enzyme was investigated at pH 7.4.

In these experiments, $AcPyADP^+$ (~6.7 mM) and AcPyADPH (~1.8 mM) were contained in the cuvette with the indicator dye PHE (10 μM), and the enzyme was added from a side arm to give a final concentration of 13.7 μM. In the first experiment, this ratio of $AcPyADP^+$ and AcPyADPH poised the potential of the solution at −0.229 V. If these analogues of NADPH had no effect on the enzyme, half-reduction of the enzyme would be expected after adding it from the side arm. However, 75% reduction was observed, on the basis of the molar absorptivities for the uncomplexed enzyme. (The molar absorptivities of the complexed enzyme and the K_d values have not been measured yet.) This corresponds to a ratio of log (ox/red) of about −0.6, which indicates a 0.018-V potential value shift in the positive direction in the presence of these analogues at these concentrations.

The potential of the solution was poised at −0.231 V in a second experiment under similar conditions. Here, 65% reduction was seen, corresponding to a 0.012-V potential value shift in the positive direction. These two experiments show that the midpoint potential value is shifted in the positive direction by 0.012–0.018 V at these concentrations of $AcPyADP^+$ and AcPyADPH. Although the K_d values for these analogues have not yet been measured, the net effect of this positive potential shift indicates a tighter binding to the reduced enzyme, thus stabilizing the reduced enzyme and making reduction easier.

A similar type of experiment was performed in the presence of 0.6 mM anthranilate with approximately 0.7 mM AcPyADPH and 9 mM $AcPyADP^+$. This ratio poised the potential of the solution at −0.218 V. After the enzyme had been added from the side arm, it appeared to be completely reduced. This potential is too negative to set up an equilibrium between the substrate-bound enzyme and these analogues.

In all three experiments, upon making the system aerobic, the absorbance at 362 nm due to AcPyADPH disappeared over a period of time, and the oxidized flavin spectrum reappeared.

Discussion

The oxidation–reduction potential studies on anthranilate hydroxylase reported here yielded information that is in striking contrast to the results obtained for two other flavoprotein hydroxylases (Einarsdottir et al., 1988; Williamson et al., 1988). For all of these enzymes, aromatic ligand-induced stimulation of enzyme reduction by NAD(P)H has been observed, and it is this phenomenon which is addressed by these electrochemical studies. While conformational changes related to binding of aromatic substrate have been described for other flavin-containing hydroxylases, they appear to be of particular importance in the "activation" of anthranilate hydroxylase for turnovers subsequent to the first (Powlowski et al., 1987b). These conformational changes might be expected to influence the redox chemistry of the enzyme.

A ratio of binding constants for oxidized and reduced flavin to the apoenzyme can be calculated from the shift in redox potential of FAD when bound to the enzyme. The midpoint potential of the enzyme-bound FAD of −0.220 V at pH 7.0 is very close to that of free flavin [−0.208 V at pH 7.0 (Draper

Table III: Dissociation Constants for Anthranilate, 3-Methylanthranilate, and Salicylate from Oxidized Anthranilate Hydroxylase, K_{ox}, and Reduced Anthranilate Hydroxylase, K_{red}[a]

	K_{ox} (M)	K_{red} (M)	K_{ox}/K_{red}
anthranilate	30×10^{-6}	18×10^{-9}	1700
3-methylanthranilate	11×10^{-4}	3.5×10^{-6}	300
		1.7×10^{-6} [b]	650
salicylate	$(150–240) \times 10^{-6}$	$(28–42) \times 10^{-9}$	5000

[a] K_{red} is calculated from K_{ox} and the shift in midpoint potential upon complexation. [b] From direct measurements.

& Ingraham, 1968)], and like free flavin, enzyme-bound FAD forms very little semiquinone. This potential value difference corresponds to a ratio of binding constants of 2.6, with the oxidized flavin bound more tightly to the apoenzyme than reduced flavin. This is a relatively small difference, and therefore any conformational change that occurs upon reduction alone appears to have a minimal effect on the interaction of the apoenzyme with flavin. In potentiometric studies, the enzyme required about 60–90 min longer to reach equilibrium in the first reduction than in the second reduction. This was seen in both the presence and the absence of the aromatic compounds studied here. The reason for this difference is unclear, although it may be related to the conformational changes undergone by the enzyme.

Binding of substrate causes the overall binding of flavin to increase by a factor of 3×10^4. In addition, the binding of substrate and/or effector allows rapid transfer of electrons by dithionite, as compared to the slow reaction of dithionite with the uncomplexed enzyme. These results support other studies that indicate the occurrence of conformational changes upon substrate binding (Powlowski et al., 1987b).

All of the aromatic ligands tested in this study have similar binding properties (Table III). Binding constants for anthranilate, the native substrate, and salicylate, an effector, differ by a factor of only 5–8, while the binding constants for these compounds for reduced enzyme differ only by a factor of 1.5–2.3. Binding of anthranilate and salicylate to the reduced form of the enzyme is 1700 and 5000 times tighter, respectively, than their binding to the oxidized enzyme. This argues for a conformational change upon enzyme reduction causing tighter binding of anthranilate and salicylate to the reduced enzyme, or better interactions with the reduced flavin of some protein residue that is freed after flavin reduction.

Results obtained with 3-methylanthranilate indicate that it binds 37 times less tightly to the oxidized enzyme than anthranilate does. The binding to the two-electron-reduced form of the enzyme is 2–3 orders of magnitude tighter than to the oxidized form of the enzyme, very similar to the trend observed with the native substrate. The trends in binding of all aromatic compounds tested are in the direction to facilitate reduction, since the reduced form of the enzyme is stabilized by binding. 3-Methylanthranilate was the only compound for which a K_d to the reduced enzyme could be measured independently, and the results agreed well with the K_d calculated from the potential shift.

It is interesting to compare these results with the redox properties of other flavoprotein hydroxylases. The midpoint potential obtained for the free enzyme is –0.220 V at pH 7.0. This is considerably more negative than the potential values reported for the two other flavoprotein hydroxylases, *p*-hydroxybenzoate hydroxylase at –0.152 V at pH 7.0 (Williamson et al., 1988) and salicylate hydroxylase at –0.077 V at pH 7.0 (Einarsdottir et al., 1988). The differing effects of substrate binding on the midpoint potentials are even more remarkable. Anthranilate shifted the midpoint potential of anthranilate hydroxylase 0.088 V in the positive direction, while the midpoint potential for *p*-hydroxybenzoate hydroxylase was shifted –0.025 V (Williamson et al., 1988) and the midpoint potential for salicylate hydroxylase was shifted –0.020 V (Einarsdottir et al., 1988) upon substrate binding. Salicylate hydroxylase and *p*-hydroxybenzoate hydroxylase bind native substrates somewhat less tightly to the reduced form of the enzyme than to the oxidized form, while anthranilate hydroxylase keeps its substrate very tightly bound in the reductive half-cycle.

The other two hydroxylases have potential values which are already significantly more positive than that of NAD(P)H/NAD(P)$^+$, while uncomplexed anthranilate hydroxylase has a more negative midpoint potential value than either of the other hydroxylases. For anthranilate hydroxylase, binding of aromatic substrate makes FAD reduction thermodynamically much more favorable. In *p*-hydroxybenzoate and salicylate hydroxylases, binding of aromatic substrate appears to play no thermodynamically important role in enhancing the rate of enzyme reduction by NAD(P)H. It is also possible, as was suggested for the other hydroxylases, that substrate binding alters the geometry between the flavin and NADPH in anthranilate hydroxylase and thus enhances the rate of reduction of the enzyme by NADPH. Indeed, it has been observed that anthranilate hydroxylase in complex with salicylate is reduced by NADPH at a rate approximately 5 times slower than that observed for the enzyme in complex with anthranilate (unpublished results). Since salicylate shifts the redox potential more than does anthranilate, anthranilate binding to the enzyme must affect more than just the redox potential in order to account for the enhanced rate of reduction by NADPH.

Like substrate binding, effector binding has opposite effects on anthranilate hydroxylase and salicylate hydroxylase. A positive potential shift of 0.101 V is seen for anthranilate hydroxylase, in contrast to the negative potential shift that has been reported for effector (benzoate) binding to salicylate hydroxylase (Einarsdottir et al., 1988). For salicylate hydroxylase, it was suggested that the nonsubstrate effector dissociates from the two-electron-reduced enzyme, leaving the enzyme uncomplexed for the oxidative half of the reaction wherein H_2O_2 is formed. However, anthranilate hydroxylase appears to be designed to bind the aromatic substrate or effector tightly in the two-electron-reduced form. Consistent with this, salicylate stabilizes a 4a intermediate in the oxidative half-reaction of anthranilate hydroxylase (Powlowski et al., 1988), whereas during oxidation of ligand-free reduced enzyme no 4a-hydroperoxide is detectable. Thus, the inability of salicylate to act as a substrate must be due to structural features affecting catalysis, not binding.

The difference in the regulation of substrate binding for anthranilate hydroxylase as compared to those for other hydroxylases may be related to the double hydroxylation carried out by this enzyme. This is a more complex reaction than those the other hydroxylases carry out, and may require a tighter binding of the aromatic compound to the two-electron-reduced enzyme. It is also tempting to speculate that substrate carries out a regulatory function since the substrate for the enzyme, anthranilate, is involved in the biodegradative pathway for tryptophan (Anderson & Dagley, 1981), as well as, presumably, the tryptophan biosynthetic pathway. The other hydroxylases are only involved in biodegradative reactions.

The effect of NADPH and NADP$^+$ analogues was examined to determine if pyridine nucleotide binding exerted a regulatory effect on anthranilate hydroxylase. Binding of NADPH and NADP$^+$ had important regulatory effects on

lipoamide dehydrogenase (Matthews et al., 1979), adrenodoxin reductase (Lambeth & Kamin, 1976), microsomal flavin monooxygenase (Beaty & Ballou, 1981), and NADPH peroxidase (Poole & Claiborne, 1986). Although binding of pyridine nucleotide to hydroxylases has been demonstrated, the importance of the regulatory role of this substrate binding on regulation and redox properties has not been investigated. Results from kinetic studies (Powlowski et al., 1988) have suggested that NADPH binding to uncomplexed enzyme may be of some importance in the first, slow, turnover of the enzyme, since the enzyme appears to be reduced more rapidly in subsequent turnovers. This suggests that the initial rate of reduction is limited by the reactions associated with the initial aromatic ligand binding.

The potential of uncomplexed anthranilate hydroxylase is shifted +0.012 to +0.018 V in the presence of AcPyADP/AcPyADPH, indicating that the reduced form of the enzyme binds the pyridine nucleotides more tightly, thus stabilizing the reduced enzyme and making reduction easier. This result is reasonable, but it indicates that the thermodynamics of electron transfer to the enzyme are not controlled significantly by the binding of pyridine nucleotide.

Registry No. Ant, 118-92-3; anthranilate hydroxylase, 37256-68-1; 3-methylanthranilate, 4389-45-1; salicylate, 69-72-7.

References

Anderson, J. J., & Dagley, S. (1981) *J. Bacteriol. 146*, 291–297.

Ballou, D. P. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) pp 301–310, Elsevier/North-Holland, New York.

Beaty, N., & Ballou, D. P. (1981) *J. Biol. Chem. 256*, 4619–4625.

Benesi, H. A., & Hildebrand, J. H. (1949) *J. Am. Chem. Soc. 71*, 2703–2707.

Draper, R. P., & Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802–808.

Duggleby, R. G. (1981) *Anal. Biochem. 110*, 9–18.

Einarsdottir, G. H., Stankovich, M. T., & Tu, S.-C. (1988) *Biochemistry 27*, 3277–3285.

Lambeth, J. D., & Kamin, H. (1976) *J. Biol. Chem. 251*, 4299–4306.

Massey, V., & Palmer, G. (1962) *J. Biol. Chem. 237*, 2347–2358.

Massey, V., Claiborne, A., Detmer, K., & Schopfer, L. (1982) in *Oxygenases and Oxygen Metabolism* (Nozaki, M., et al., Eds.) pp 185–195, Academic Press, New York.

Matthews, R. G., Ballou, D. P., & Williams, C. H. (1979) *J. Biol. Chem. 254*, 4974–4981.

McIlwain, H. (1937) *J. Chem. Soc. 2*, 1704–1711.

Orr, G. A., & Blanchard, J. S. (1984) *Anal. Biochem. 142*, 232–234.

Poole, L. B., & Claiborne, A. (1986) *J. Biol. Chem. 261*, 14525–14533.

Powlowski, J. B., Ballou, D. P., & Massey, V. (1985) *Biochemistry 24*, 3379 (Abstract 129).

Powlowski, J. B., Dagley, S., Massey, V., & Ballou, D. B. (1987a) *J. Biol. Chem. 262*, 69–74.

Powlowski, J. B., Ballou, D. P., & Massey, V. (1987b) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 565–568, de Gruyter, Berlin.

Powlowski, J. B., Massey, V., & Ballou, D. P. (1988) *J. Biol. Chem.* (submitted for publication).

Ryerson, C. C., Ballou, D. P., & Walsh, C. (1982) *Biochemistry 21*, 1144–1151.

Stankovich, M. T. (1980) *Anal. Biochem. 109*, 295–308.

Stankovich, M., & Fox, B. (1983) *Biochemistry 22*, 4466–4472.

Williamson, G., Edmondson, D. E., & Muller, F. (1988) *Biochim. Biophys. Acta 953*, 258–262.