Studies of Electron-Transfer Properties of Salicylate Hydroxylase from *Pseudomonas cepacia* and Effects of Salicylate and Benzoate Binding†

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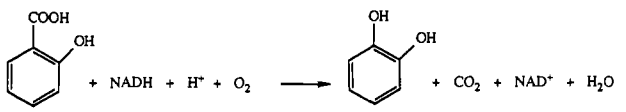
ABSTRACT: The pH dependence of the redox behavior of salicylate hydroxylase from *Pseudomonas cepacia* as well as the effects of salicylate, benzoate, and chloride binding is described. At pH 7.6 in 0.02 M potassium phosphate buffer $E_1°'$ ($EFl_{ox}/EFl^{\bullet-}$) is −0.150 V and $E_2°'$ ($EFl^{\bullet-}/EFl_{red}H^-$) is −0.040 V versus the standard hydrogen electrode (SHE). A maximum of 5% of FAD anion semiquinone is thermodynamically stabilized under these conditions. However, in coulometric and dithionite titrations more semiquinone is kinetically formed, indicating slow transfer of the second electron. The potential/pH dependence is consistent with a two-electron, one-proton transfer. Upon salicylate binding the midpoint potential is shifted 0.020 V negative from −0.094 to −0.114 V vs SHE at pH 7.6. A maximum of 7% of the neutral semiquinone is stabilized both in potentiometric and coulometric titrations. This small potential shift indicates that the substrate is bound nearly to the same extent to all three oxidation states of the enzyme. It is clear that the substrate binding does not make the reduction of the flavin thermodynamically more favorable. In contrast to salicylate, the potential shift caused by the effector, benzoate, is much more significant. (A maximum potential shift of −0.07 V is calculated.) Benzoate binds most tightly to the oxidized form and is least tightly bound to the two-electron-reduced form of the enzyme. For the reduction of the free enzyme the transfer of the second electron or the transfer of the proton is rate limiting, as is shown by the kinetic formation of the anionic semiquinone. Binding of an aromatic substrate/effector keeps the flavin in a configuration in both the oxidized and one-electron-reduced states that allows for fast transfer of the proton and both the first and second electron, thus enhancing the rate of reduction. The salicylate substrate remains tighly bound to the fully reduced enzyme, allowing for the subsequent hydroxylation, whereas the benzoate effector tends to dissociate from the fully reduced enzyme, leading to H_2O_2 formation.

Salicylate hydroxylase (EC 1.14.13.1), an FAD[1]-dependent external monooxygenase, catalyzes the decarboxylative hydroxylation of salicylate to form CO_2, catechol, and water with stoichiometric consumption of NADH and O_2.



This enzyme was first isolated from *Pseudomonas putida* (Katagiri et al., 1962; Yamamoto et al., 1965) and later from strains of soil microorganisms, *Pseudomonas* sp. ATCC 29352 (White-Stevens & Kamin, 1972; White-Stevens et al., 1972), *Pseudomonas* sp. ATCC 29351 (Presswood & Kamin, 1976), *Pseudomonas cepacia* (Tu et al., 1981; Wang & Tu, 1984), and *Trichosporon cutaneum* (Sze & Dagley, 1984). The *P. putida* and *T. cutaneum* enzymes are quite different from the other hydroxylases. The salicylate hydroxylases from *P.* sp. ATCC 29352, *P.* sp. ATCC 29351, and *P. cepacia* are very similar; they have the same molecular weight and subunit structure as well as substrate specificities. However, significant differences in certain kinetic and spectral properties have been observed. When salicylate hydroxylase from *P. cepacia* is complexed with salicylate, the rate of enzyme reduction by NADH is enhanced 290-fold compared to the reduction of the free enzyme by NADH (Wang & Tu, 1984). The flavoenzyme is reduced, and NAD^+ is then released. Subsequently, the reduced enzyme–salicylate complex reacts with oxygen to form the products. The enzyme binds salicylate and NADH randomly. For salicylate hydroxylase from *P. putida* (Takemori et al., 1972) and *P.* sp. ATCC 29351 (Presswood & Kamin, 1976), it has been shown that salicylate is bound relatively tightly to both the oxidized and the reduced enzyme, thus preventing dissociation of the salicylate from the reduced enzyme–salicylate complex before it reacts with oxygen. Benzoate also enhances the rate of enzyme reduction by NADH with a subsequent formation of H_2O_2 (oxidase activity). Benzoate is not hydroxylated in the reaction and remains unchanged, acting as an effector. Monovalent anions, like chloride, have been shown to act as competitive inhibitors with respect to salicylate (White-Stevens et al., 1972).

Upon dithionite or photochemical reduction of free salicylate hydroxylase from *P. putida* (Takemori et al., 1969) the anionic semiquinone is formed, and when salicylate hydroxylase is complexed with salicylate, an EPR-silent species having

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[1] Abbreviations: 9AzaRf, 9-azariboflavin; 8ClRf, 8-chlororiboflavin; Benz, benzoate; EFl_{ox}, oxidized salicylate hydroxylase; $EFl^{\bullet-}$, anionic semiquinone salicylate hydroxylase; $EFl_{red}H^-$, two-electron-reduced salicylate hydroxylase; EFl_{ox}Benz, benzoate-bound enzyme; $EFl_{red}H^-$Benz, two-electron-reduced benzoate-bound enzyme; EFl_{ox}Sal, salicylate-bound enzyme; $EFlH^{\bullet}$Sal, neutral semiquinone salicylate bound enzyme; $EFl_{red}H^-$Sal, two-electron-reduced salicylate-bound enzyme; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; 2HNQ, 2-hydroxy-1,4-naphthoquinone; IDS, indigodisulfonate; $MV^{\bullet+}$, one-electron-reduced methylviologen; Sal, salicylate.

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spectral properties similar to those of the neutral semiquinone is formed. Similar observations have been observed upon photoreduction of the *P.* sp. ATCC 29352 enzyme (White-Stevens et al., 1972), and for the *P.* sp. ATCC 29352 enzyme, the anionic semiquinone was observed upon photoreduction of the enzyme in the presence of benzoate.

Although substrate binding markedly affects the kinetics of NADH reduction and the form of flavin semiquinone stabilized by salicylate hydroxylase, the binding constants of substrate for both oxidized and reduced enzyme are very similar. This predicts that the redox properties of salicylate hydroxylase would show little change upon substrate binding. We wanted to further clarify the role of thermodynamics in the control of salicylate hydroxylase isolated from *P. cepacia*. Using spectroelectrochemistry as a tool, we have examined the effect of salicylate binding on the thermodynamics of electron transfer to FAD in the active site and compared these effects with those resulting from benzoate binding. The effect of chloride binding is also addressed.

We present here the results of the first determination of the redox potentials on a flavoprotein hydroxylase. From redox studies of free, substrate-bound, effector-bound, and inhibited enzyme, redox potential values and the amount of semiquinone thermodynamically stabilized were obtained. From the potential shifts, relative and absolute binding constants to the oxidized, the one-electron-reduced, and the two-electron-reduced enzyme were obtained. From examination of these binding constants and the thermodynamic amount of semiquinone stabilized, differences in behavior have been distinguished for free, substrate-bound, and effector-bound ezyme.

Materials and Methods

Materials. Salicylate hydroxylase was isolated from *Pseudomonas cepacia* according to the method of Tu et al. (1981). Glass-distilled water was used in all experiments. Sodium salicylate from EM Science (Gibbstown, NJ) was recrystallized from hot water. Methylviologen was purchased from British Drug House (Poole, England). Indigodisulfonate was purchased from Matheson Coleman and Bell. 9-Azariboflavin was the generous gift of Dr. D. Graham at Merck, Sharp and Dohme. Sodium benzoate 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).

Methods. Coulometric titrations were performed as previously described (Stankovich, 1980; Stankovich & Fox, 1983). The concentrations of free salicylate hydroxylase, salicylate-bound enzyme, benzoate-bound enzyme, and chloride-inhibited enzyme were determined by using molar absorptivities of 11 300, 12 000, 12 000, and 10 200 M^{-1} cm^{-1} at their wavelengths of maximum absorbance, 450, 446, 448, and 450 nm, respectively. The enzyme was dialyzed against two changes of the buffer before each experiment. All experiments were done in 0.02 M potassium phosphate buffer at 10 °C unless otherwise stated. All electrochemical experiments contained 50 μM methylviologen as a mediator titrant to facilitate reduction.

Dithionite experiments were performed in a manner similar to the method of Foust et al. (1969), and the dithionite solution used was standardized with lumiflavin-3-acetate, which was the generous gift of Dr. S. Ghisla, University of Konstanz, FRG.

Potentiometric titrations were performed as described (Stankovich, 1980; Stankovich & Fox, 1983). In control experiments all redox mediator dyes were titrated individually to obtain the redox potentials and spectral characteristics. This enabled the absorbance of each of the indicators to be calculated and subtracted, leaving only the absorption spectrum of the enzyme. Potential values were measured at one pH by using 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. The potentials of the individual electrons were calculated from the equations

$$E_1^{o\prime} - E_2^{o\prime} = 0.11236 \log [2M/(1 - M)] \quad (1)$$

$$E_1^{o\prime} + E_2^{o\prime} = 2E_m \quad (2)$$

where M is the maximum fraction of semiquinone formed, obtained from absorbance and EPR data. Note that the subscripting is opposite that used by Clark (1960). All potential values are reported versus SHE.

The ratio of binding constants for salicylate binding to the enzyme was calculated from the equation

$$E_{bound} = E_m - \frac{0.056}{n} \log \frac{K_{a1}}{K_{a2}} \quad (3)$$

where E_{bound} is the midpoint potential for salicylate-bound enzyme, E_m is the midpoint potential for the free enzyme,

$$K_{a1} = [EFl_{ox}Sal]/[EFl_{ox}][Sal] \quad (4)$$

and

$$K_{a2} = [EFl_{red}H^-Sal]/[EFl_{red}H^-][Sal] \quad (5)$$

When benzoate binds to the salicylate hydroxylase, it cannot be assumed that all forms of the enzyme are saturated. To correct for the nonsaturation of the reduced enzyme in the presence of benzoate, K_{d2} was calculated from the equation

$$E_{Benz} = E_m + \frac{0.056}{n} \log \frac{1 + ([Benz]/K_{d2})}{1 + ([Benz]/K_{d1})} \quad (6)$$

where E_{Benz} is the midpoint potential measured for the enzyme at a specified benzoate concentration ([Benz]),

$$K_{d1} = [EFl_{ox}][Benz]/[EFl_{ox}Benz] \quad (7)$$

and

$$K_{d2} = [EFl_{red}H^-][Benz]/[EFl_{red}H^-Benz] \quad (8)$$

Electron paramagnetic resonance (EPR) measurements were made on a Varian 109 spectrometer in the laboratory of Dr. John D. Lipscomb at the University of Minnesota. A modification of the procedure of Beinert (Beinert et al., 1978) was used to maintain anaerobicity. For the free enzyme, 87 μM salicylate hydroxylase and 8 μM indigodisulfonate were reduced with 2.5 mM dithionite in 0.02 M potassium phosphate at pH 8.0. The presence of IDS enabled the kinetic barriers to be overcome such that the thermodynamic amount of anionic semiquinone would be stabilized. For the salicylate-bound enzyme, 187 μM enzyme in the presence of 2 mM sodium salicylate was reduced with excess dithionite and titrated oxidatively with 3 mM potassium ferricyanide in 0.02 M potassium phosphate at pH 7.0. The experiments were carried out under argon atmosphere. The instrument conditions were as follows: temperature, 20 K; microwave power, 0.06 mW; microwave frequency, 9.22 GHz; free enzyme g-factor, 2.001, salicylate-bound enzyme g-factor, 2.002. A standard, 1 mM $CuClO_4$, was used to quantitate the amount of semiquinone formed. Visible spectra of the enzyme in the EPR tube were recorded by a Hewlett-Packard 8451A diode array spectrophotometer. The cell path length of the EPR tube

Table I: Redox Potential Data on Salicylate Hydroxylase at 10 °C

pH	redox indicator	E_m indicator (V)	concn of indicator (μM)	enzyme concn (μM)	E_m enzyme (V)	slope (V)	semiquinone stabilized (%)	no. of points
6.2	IDS	−0.028	1.3	8.3	−0.059 (R)[a]	0.028	0[b]	2
6.8	IDS	−0.047	3.4	11.7	−0.068 (R)	0.036	0	7
6.8	2HNQ	−0.082	1.5	9.3	−0.073 (R)	0.035	0	5
7.0	IDS	−0.060	4.5	23.1	−0.075 (R)	0.032	0	5
7.0[c]	IDS	−0.086	2.0	10.3	−0.080 (R)	0.029	0	5
7.2	IDS	−0.068	1.7	7.3	−0.085 (R)	0.033	0	5
7.6	IDS	−0.089	3.2	14.1	−0.093 (R)	0.033	4	6
7.6	IDS	−0.089	3.2	14.1	−0.092 (O)	0.036	4	6
7.6	IDS	−0.089	2.8	10.9	−0.095 (R)	0.036	7	5
8.0	IDS	−0.098	1.8	8.0	−0.104 (R)	0.039	12	6

[a] (R) and (O) indicate potentiometric titrations performed in reductive (R) and oxidative (O) directions. [b] No semiquinone detected. [c] 0.100 M potassium phosphate buffer; all other experiments in 0.020 M potassium phosphate buffer.

was determined to be 0.272 cm (data not shown). The sample in the EPR tube was sonicated for a very short time after thawing to remove bubbles formed upon freezing and thawing, which cause aberrations in the absorption spectrum.

Results

Dithionite Titrations of Free Enzyme. When salicylate hydroxylase was reduced with sodium dithionite at pH 8.0, a species that has an absorption spectrum characteristic of the anionic flavin semiquinone (Massey & Palmer, 1966) was observed as shown in Figure 1. The conversion to the semiquinone proceeded isosbestically at 500 and 406 nm, and the maximum amount of semiquinone was observed upon addition of about 1 equiv of electrons beyond that required to remove initial oxygen. As the second reductive equivalent is transferred to salicylate hydroxylase, the isosbestic points are broken, and the two-electron-reduced enzyme is formed. Dithionite titrations yield the value of n (number of electrons transferred) as well as molar absorptivities of reduced enzyme. The molar absorptivities obtained for the fully reduced enzyme at 450 and 370 nm were 1500 and 5400 M^{-1} cm^{-1}, respectively. An estimate of the molar absorptivities for the semiquinone form can be obtained. The inset of Figure 1 is a plot of absorbance versus n at 450 and 370 nm. The largest relative differences in molar absorptivities occur at these wavelengths. The intersections of the lines at $n = 1$ yields the absorbance values for 100% semiquinone formation from which molar absorptivities of 16 800 and 4350 M^{-1} cm^{-1} were obtained at 370 and 450 nm, respectively. Molar absorptivities obtained from a plot of absorbance at 370 nm vs absorbance at 450 nm are in good agreement with those described above. By use of these molar absorptivities and the total enzyme concentration, the maximum amount of the anionic semiquinone stabilized at half-reduction was calculated to be 80% at pH 8.0. In a separate dithionite titration at pH 7.6, a maximum of 75% anionic semiquinone formation was observed.

Coulometric Titrations of Free Enzyme. Coulometric titrations of the free enzyme yielded considerably less anionic semiquinone than dithionite titrations. A maximum of 25%, 35%, and 75% of the anionic semiquinone was observed at pH values 6.8, 7.6, and 8.0, respectively. At pH 8.0 transfer of electrons to the flavin semiquinone becomes more difficult than at the other pH values, and significantly more of the anionic semiquinone is formed at this pH. When the titration was approximately half completed, absorbance due to $MV^{\bullet+}$ began to appear in the spectra, whereas at lower pH values absorbance due to $MV^{\bullet+}$ did not appear in the spectra until the end of the titrations. In the second half of the titration at pH 8.0, electron transfer required approximately 90 min/aliquot of charge, whereas at lower pH values electron transfer to the enzyme required less than 1 min/aliquot of charge. Slow electron transfer between $MV^{\bullet+}$ and semiquinone has been observed with glycolate oxidase (Pace & Stankovich, 1986) and D-amino-acid oxidase (Van den Berghe-Snorek & Stankovich, 1985).

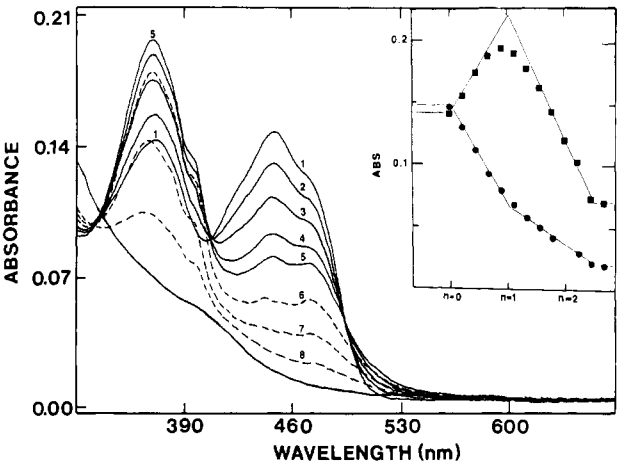


FIGURE 1: Dithionite reduction of salicylate hydroxylase, 13 μM enzyme in 0.02 M potassium phosphate buffer, pH 8.0, 10 °C. Concentration of dithionite was 1.4 mM. (1) Oxidized enzyme; (2) $n = 0.2$; (3) $n = 0.4$; (4) $n = 0.6$; (5) $n = 0.8$; (6) $n = 1.2$; (7) $n = 1.5$; (8) $n = 1.9$; (9) excess dithionite. The inset figure shows the absorbance vs number of reducing equivalents added plotted for (●) 450 and (■) 370 nm.

As shown by the potentiometric titrations described below, the percentages of semiquinone obtained in dithionite and coulometric titrations are not equilibrium values. For example, a maximum of 12% of salicylate hydroxylase was present as the anionic semiquinone during potentiometric titrations at pH 8.0, whereas a maximum 75% and 80% anionic semiquinone was obtained during coulometric and dithionite titrations. The high percentage of semiquinone observed during dithionite and coulometric titrations must be due to kinetic barriers that are overcome in the presence of redox indicator dyes that exist in redox equilibrium with the enzyme. Similar observations have been reported for glucose oxidase (Stankovich et al., 1978) and glycolate oxidase (Pace & Stankovich, 1986).

Potentiometric Titrations of Free Enzyme. The pH dependence of electron transfer was determined over the pH range 6.2–8.0. Results for the midpoint potential values for the free enzyme and indicator dyes used are summarized in Table I. The midpoint potential values for IDS are more positive than the published values, and the potential/pH behavior is not ideal. This could possibly be due to impurities with higher potential values in the dye or the difference in temperature and buffer concentration. The results at pH 7.6

for the free enzyme will be presented first since the reversibility of the electron transfer was tested at this pH. Salicylate hydroxylase and the redox indicator dye used, IDS, reduced concurrently. Thermodynamic stabilization of a small amount of the anionic semiquinone was observed when spectral contributions of IDS were subtracted. A midpoint potential (E_m) of −0.093 V was obtained in the reductive direction and an E_m of −0.092 V in the oxidative direction. Plots of measured potential versus logarithm ($EFl_{ox}/EFl_{red}H^-$) gave slopes of 0.033 and 0.036 V in the reductive and oxidative directions, respectively, both of which are near the Nernstian value of 0.028 V for a two-electron transfer at 10 °C. This clearly shows the reversibility of the electron transfer. Another reductive potentiometric titration gaven an E_m of −0.095 V. On average, about 5% of the semiquinone was stabilized at half-reduction in these titrations at pH 7.6 as shown in Table I. On the basis of the amount of semiquinone stabilized, the potential values for the individual electrons were calculated.

$$EFl_{ox} + e^- \rightleftharpoons EFl^{\bullet -}$$

$$E_1^{\circ\prime} = -0.150 \text{ V vs SHE} \quad (9)$$

$$EFl^{\bullet -} + e^- + H^+ \rightleftharpoons EFl_{red}H^-$$

$$E_2^{\circ\prime} = -0.040 \text{ V vs SHE} \quad (10)$$

A potentiometric titration of salicylate hydroxylase at pH 6.8, using IDS as the redox mediator, gave an E_m of −0.068 V with a Nernst slope of 0.036 V. A separate potentiometric titration under the same conditions using 2HNQ as the redox mediator gave an E_m of −0.073 V with a Nernst slope of 0.035 V. These two midpoint potential values agree within 0.005 V and indicate that the midpoint potential is independent of the type of redox indicator used. At pH 6.8 no thermodynamic stabilization of the anionic semiquinone could be spectrally detected.

An EPR experiment was performed to verify that a small amount of the anionic semiquinone was thermodynamically stabilized. The experiment was performed at pH 8.0, where stabilization of the anionic semiquinone is greatest over the pH range studied. A maximum of 6% of the anionic semiquinone was obtained. The value from the EPR experiment was within 6% of the value obtained spectrophotometrically (12%). There is also an inherent error of 5–10% in quantitative EPR experiments, so the agreement of the two types of experiments at pH 8.0 is reasonable.

The potential separation of the individual electrons was not calculated at pH values other than 7.6. This is due to lack of semiquinone stabilization at the lower pH values (see Table I) and the high relative error in the measurement at pH 8.0. However, the trend in the amount of semiquinone stabilized, i.e., more semiquinone stabilized at high pH, is consistent with the potential values and protonation assignment shown in the electron-transfer equations (eq 9, 10) shown above. The potential value of the second electron is more positive than the potential value of the first electron but becomes more negative at higher pH, thus approaching the potential of the first electron.

Increasing the concentration from 0.02 to 0.10 M potassium phosphate at pH 7.0 had no significant effect on the midpoint potential of the free enzyme (see Table I).

Figure 2, line A, shows the potential/pH behavior for salicylate hydroxylase over the pH range 6.2–8.0. There is a 0.026 V potential/pH unit change, which is consistent with a two-electron, one-proton transfer. The proton is assumed to be transferred with the second electron since it is the anionic semiquinone that is stabilized.

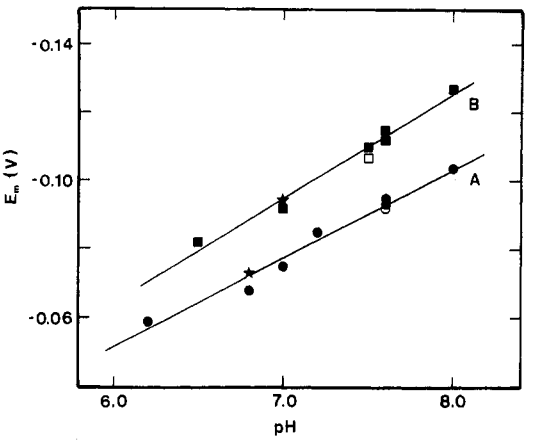


FIGURE 2: Potential vs pH behavior of salicylate hydroxylase in 0.02 M phosphate buffer at 10 °C. Line A represents the free enzyme and line B the enzyme in the presence of 0.2 mM salicylate. Redox mediator dyes used were (●) IDS (reductive direction), (○) IDS (oxidative direction), (★) 2HNQ (reductive direction), (■) 9AzaRf (reductive direction), and (□) 9AzaRf (oxidative direction).

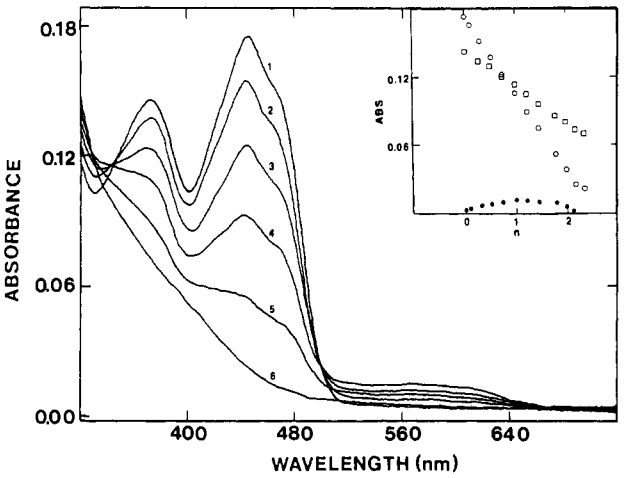


FIGURE 3: Coulometric titration of salicylate-bound salicylate hydroxylase, 14.4 μM enzyme, 0.2 mM sodium salicylate, and 50 μM methylviologen in 0.02 M potassium phosphate buffer, pH 7.0 at 10 °C. Ten percent of the neutral semiquinone was stabilized. (1) Oxidized enzyme; (2) n = 0.36; (3) n = 0.72; (4) n = 1.21; (5) n = 1.80; (6) n = 2.1. The inset figure shows the absorbance vs number of reducing equivalents added plotted for (●) 570, (○) 446, and (□) 376 nm. Current efficiency was 94%.

Titrations of Salicylate-Bound Salicylate Hydroxylase. When salicylate hydroxylase was electrochemically reduced in the presence of 0.2 mM sodium salicylate at pH 7.0, a long-wavelength-absorbing species was observed as shown in Figure 3. This long-wavelength absorbance increases in the first half of the titration and then decreases in the second half. This long-wavelength absorbance is characteristic of the neutral flavin semiquinone (Massey & Palmer, 1966) with the maximum absorbance occurring at 570 nm. When the substrate-bound enzyme is reduced with dithionite, the same amount of semiquinone is formed; however, when the fully reduced substrate enzyme complex is oxidized with ferricyanide, considerably more of the semiquinone is formed. Greater stabilization of the neutral semiquinone upon ferricyanide oxidation has also been observed with the neutral semiquinone of general acyl-CoA dehydrogenase (Thorpe et al., 1979). As shown in Figure 4, the long-wavelength-absorbing species is EPR active, verifying the presence of the neutral semiquinone. This is in contrast to the previous work on *P. putida* salicylate hydroxylase (Takemori et al., 1969) in which no EPR signal was seen for the long-wavelength-

Table II: Redox Potential Data on Bound Salicylate Hydroxylase

pH	redox indicator	E_m indicator (V)	concentration indicator (μM)	concentration enzyme (μM)	E_m enzyme (V)	slope (V)	semiquinone stabilized (%)	$E_1^{\circ\prime}$[a] enzyme (V)	$E_2^{\circ\prime}$[a] enzyme (V)	no. of points
				Salicylate-Bound Salicylate Hydroxylase (0.2 mM)						
6.5	9AzaRf	−0.080	1.6	8.3	−0.082 (R)[b]	0.036	15	−0.107	−0.057	5
7.0	9AzaRf	−0.106	2.3	25.6	−0.092 (R)	0.033	12	−0.124	−0.060	7
7.0	2HNQ	−0.093	2.0	17.0	−0.094 (R)	0.037	11	−0.128	−0.060	6
7.5	9AzaRf	−0.111	1.3	11.0	−0.110 (R)	0.036	8	−0.153	−0.067	6
7.5	9AzaRf	−0.111	1.3	11.0	−0.107 (O)	0.039	8	−0.150	−0.064	6
7.6	9AzaRf	−0.115	2.1	18.5	−0.112 (R)	0.035	8	−0.155	−0.069	4
7.6	9AzaRf	−0.115	2.5	13.8	−0.115 (R)	0.028	6	−0.166	−0.064	7
8.0	9AzaRf	−0.127	1.4	19.6	−0.127 (R)	0.033	5	−0.182	−0.072	7
				Salicylate Hydroxylase in the Presence of Benzoate						
7.6[c]	9AzaRf	−0.122	1.0	13.4	−0.129 (R)	0.032	0[d]			8
7.6[c]	9AzaRf	−0.122	1.3	10.5	−0.125 (R)	0.034	0			7
7.6[e]	8ClRf	−0.157	1.8	11.9	−0.147 (R)	0.030	0			7
7.6[e]	8ClRf	−0.157	0.9	8.0	−0.148 (R)	0.032	0			5
				Salicylate Hydroxylase Inhibited with Chloride (0.5 M)[f]						
7.6	8ClRf	−0.156	1.8	10.5	−0.178 (R)	0.036	0			7
7.6	8ClRf	−0.156	1.6	9.8	−0.182 (R)	0.032	0			8

[a] $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ calculated from the amount of semiquinone stabilized. [b] (R) and (O) indicate potentiometric titrations performed in reductive (R) and oxidative (O) directions. [c] Benzoate concentration 0.035 M. [d] No semiquinone detected. [e] Benzoate concentration 0.2 M. [f] Sodium salicylate concentration 0.2 mM.

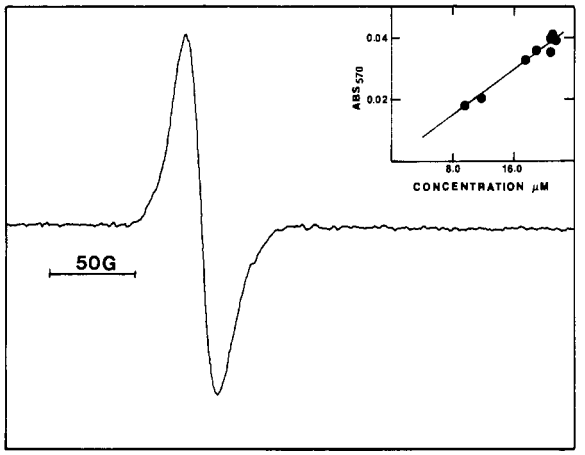


FIGURE 4: EPR spectra of the neutral semiquinone form of the salicylate-bound salicylate hydroxylase; line width 21 G. Conditions: 173 μM enzyme was reduced with excess sodium dithionite and titrated oxidatively with 3 mM potassium ferricyanide in 0.02 M potassium phosphate, pH 7, 2 mM sodium salicylate. The inset figure shows a plot of A_{570} vs concentration of semiquinone. The absorbance was measured in the EPR tube, path length 0.272 cm. From the slope of the line of the points shown a molar absorptivity of 6600 M^{-1} cm^{-1} was obtained at 570 nm.

absorbing species of substrate-bound salicylate hydroxylase. The molar absorptivity for the neutral semiquinone was obtained from the EPR titration. The inset of Figure 4 shows a plot of absorbance at 570 nm vs concentration of semiquinone formed at each point in the titration. The molar absorptivity obtained this way is 6600 M^{-1} cm^{-1} by Beer's law. This molar absorptivity is higher than what has been reported previously for neutral flavin semiquinones, where the molar absorptivities range from 3000 to 6000 M^{-1} cm^{-1} (Massey & Palmer, 1966; Barman & Tollin, 1972; Thorpe et al., 1979). The maximum amounts of neutral semiquinone observed in coulometric and dithionite titrations were within 2% of the amounts stabilized under thermodynamic conditions described below.

Potentiometric Titrations of Salicylate-Bound Salicylate Hydroxylase. The potential/pH dependence of the salicylate-bound salicylate hydroxylase was determined over the pH range 6.5–8.0. Results for the midpoint potential values for the salicylate-bound enzyme and redox indicator dyes used are summarized in Table II. Also included in Table II are the amount of radical stabilized and the E_1° and $E_2^{\circ\prime}$ values calculated from the amount of semiquinone stabilized at each pH. Figure 2, line B, shows the potential/pH behavior for salicylate-bound enzyme over the pH range 6.5–8.0. There is a 0.030 V potential/pH change, which again is consistent with a two-electron, one-proton transfer.

The reversibility of the electron transfer for the substrate-bound enzyme was tested at pH 7.5 by using 9AzaRf as a redox indicator. A midpoint potential of −0.110 V was obtained in the reductive direction with a Nernst slope of 0.036 V, while a midpoint of −0.107 V was obtained in the oxidative direction with a Nernst slope of 0.039 V. These results indicate a reversible electron transfer. The same amount of the neutral semiquinone is stabilized in both directions. The data presented in Table II also show that the substrate-bound enzyme does not bind to the redox indicator dyes. Two potentiometric titrations at pH 7.0 using two different redox indicator dyes (9AzaRf and 2HNQ) gave midpoint potentials that agreed within 0.002 V (see Table II). At pH 7.6 the average E_m value was −0.114 V. This is 0.020 V more negative than the midpoint potential for the free enzyme. This small potential shift indicates that the ratio of binding constants for salicylate to the oxidized and reduced enzyme is 5, with salicylate being more tightly bound to the oxidized form of the enzyme. On the basis of the amount of neutral semiquinone stabilized, the potential for the individual electrons can be calculated.

$$EFl_{ox}Sal + e^- + H^+ \rightleftharpoons EFlH^{\bullet}Sal$$

$$E_1^{\circ\prime} = -0.160 \text{ V vs SHE} \quad (11)$$

$$EFlH^{\bullet}Sal + e^- \rightleftharpoons EFl_{red}H^-Sal$$

$$E_2^{\circ\prime} = -0.067 \text{ V vs SHE} \quad (12)$$

When salicylate is bound to the enzyme, the proton is transferred with the first electron, while the proton is transferred with the second electron for the free enzyme. At all pH values studied the neutral semiquinone was stabilized for the salicylate-bound enzyme, with more semiquinone thermodynamically stabilized at the lower pH values.

Figure 5 shows the potential/pH behavior for the individual electrons as calculated from the midpoint potential and the amount of semiquinone stabilized. At all pH values studied,

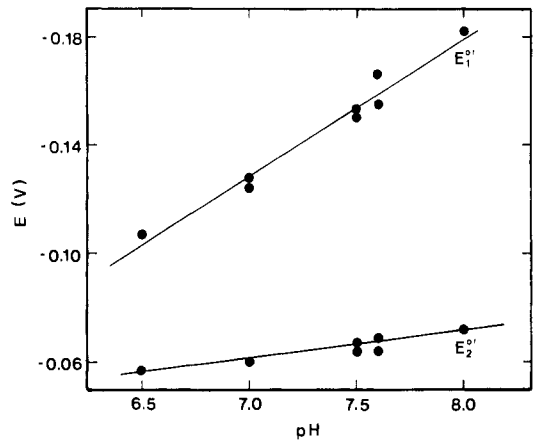


FIGURE 5: Potential vs pH behavior for the individual electrons for salicylate-bound salicylate hydroxylase in 0.020 M potassium phosphate and 0.2 mM sodium salicylate as calculated from the midpoint potential values and the maximum thermodynamic amount of semiquinone stabilized.

$E_1^{0'}$ is more negative than $E_2^{0'}$, and $E_1^{0'}$ has a 0.051 V potential/pH change, which is close to the ideal 0.056 V/pH for one-proton, one-electron transfer at 10 °C. The potential/pH dependence for $E_2^{0'}$ is 0.010 V. The second electron is nearly pH independent with an average value of −0.064 V. The nonideal potential/pH dependence could result from error in the molar absorptivity for the neutral semiquinone. A very small amount of neutral semiquinone is thermodynamically stabilized in these experiments, ranging from 5% to 15% semiquinone at half reduction. The potential separation is a logarithmic function of the amount of radical stabilized. Therefore, a relatively small error in the determination of the amount of semiquinone stabilized causes a larger error in the potential separation calculated.

Effects of Benzoate Binding. Most studies on benzoate complexed to salicylate hydroxylase have been done with a benzoate concentration of 0.030 M, which is about 15 times the K_d for benzoate bound to the oxidized enzyme (White-Stevens et al., 1972). Midpoint potentials obtained for salicylate hydroxylase complexed to benzoate at 0.035 M benzoate were −0.125 and −0.129 V with 0.034- and 0.032-V Nernst slopes, respectively, as shown in Table II. No semiquinone formation was detected.

Increasing the benzoate concentration to 0.2 M, where some effector inhibition occurs, shifted the midpoint potential 0.054 V negative of that of the free enzyme and 0.022 V negative of that of the enzyme in 0.035 M benzoate. Midpoint potentials of −0.147 and −0.148 V were obtained with 0.030- and 0.032- V Nernst slopes, respectively, as shown in Table II.

By use of these midpoint potential values for the two concentrations of benzoate in eq 6 and a value of 0.0023 M for K_{d1} (White-Stevens & Kamin, 1972), the dissociation constant for benzoate to the two-electron-reduced enzyme, K_{d2}, was calculated to be ~0.4 M at 0.035 M benzoate and ~0.8 M at 0.2 M benzoate. As previously stated for the calculations of potential separation for the individual electrons for the salicylate-bound enzyme, the K_d values are logarithmic functions of potential. Thus, the calculated K_{d2} values are very sensitive to small changes in potential. These calculated K_d values clearly show that under these experimental conditions only a small fraction of the two-electron-reduced form of salicylate hydroxylase is bound to benzoate.

When salicylate hydroxylase was electrochemically reduced in the presence of 0.035 M benzoate with no dyes present, no semiquinone could be detected. Reductive dithionite and oxidative ferricyanide titrations were performed to test for semiquinone formation. In the dithionite titration the anionic semiquinone was formed as shown in Figure 6A. On the other hand, in 0.2 M benzoate no semiquinone stabilization was seen as shown in Figure 6B. The anionic semiquinone observed at 0.035 M benzoate is most likely the anionic semiquinone for the free enzyme. Kinetic formation of the anionic semiquinone was observed in dithionite reductions for the free enzyme. In 0.035 M benzoate the one-electron-reduced enzyme is not saturated, but it is saturated in 0.2 M benzoate. This indicates that the one-electron-reduced enzyme binds benzoate less tightly than the oxidized enzyme. The anionic semiquinone was also seen upon photochemical reduction of benzoate-complexed *P.* sp. ATCC 29352 under similar conditions (White-Stevens et al., 1972). In the ferricyanide titration no semiquinone was observed.

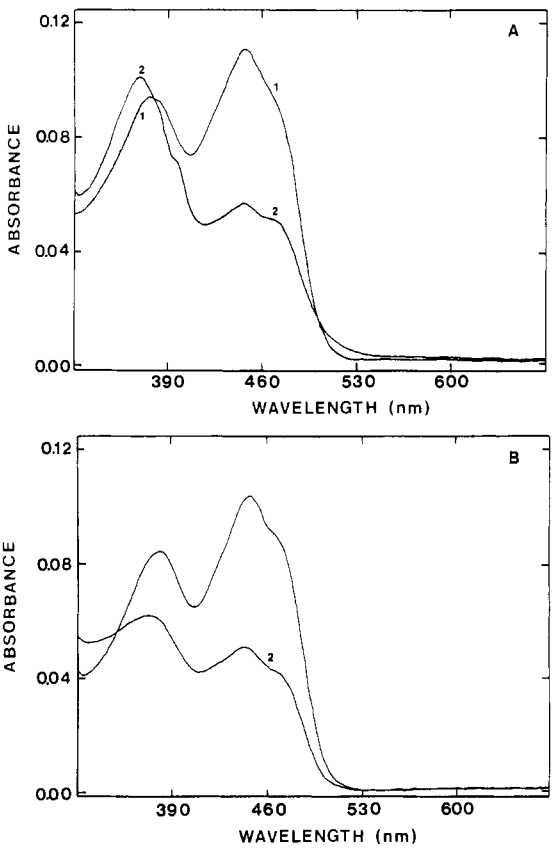
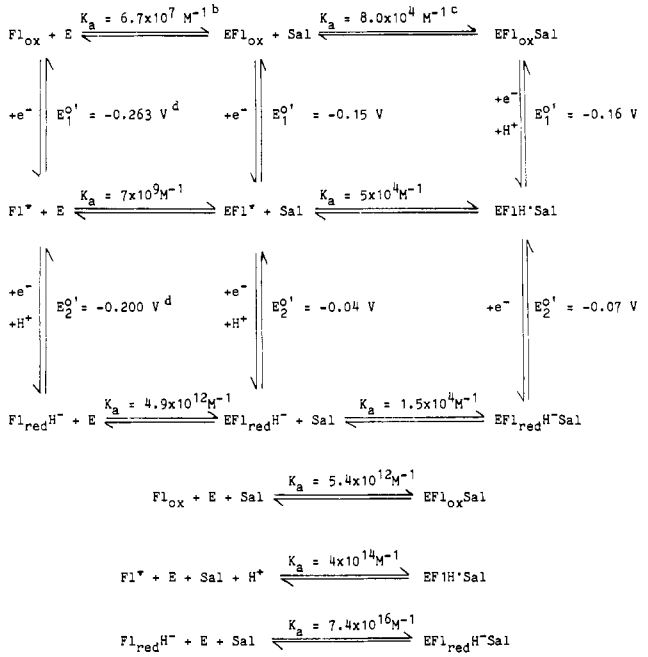


FIGURE 6: (A) 9.2 μM salicylate hydroxylase in 0.02 M potassium phosphate and 0.035 M sodium benzoate, pH 7.6 at 10 °C. (1) Oxidized spectrum; (2) after 0.9 electron equivalent of sodium dithionite was added. (B) 8.6 μM salicylate hydroxylase in 0.02 M potassium phosphate and 0.2 M sodium benzoate, pH 7.6 at 10 °C. (1) Oxidized spectrum; (2) after 1.2 electron equivalents of sodium dithionite was added.

Effect of Chloride on the Redox Potential. In 0.5 M chloride and 0.2 mM salicylate the spectrum of the enzyme resembles much more that of the free enzyme than that of the substrate-bound enzyme. The maximum absorbance is still at 450 nm but with a lower molar absorptivity. Upon electrochemical reduction neither neutral nor anionic forms of the semiquinone could be spectrally identified. Two potentiometric titrations were done at pH 7.6. The midpoint potential values were −0.178 and −0.182 V with 0.036- and 0.032-V Nernst slopes, respectively (see Table II). Compared to the midpoint potential of the free enzyme, the midpoint potential is shifted 0.086 V negative. However, this value is still positive of the midpoint potential for the NADH/NAD^+ couple (E_m = −0.320 V, pH 7.0). It was observed that NADH is still capable

Scheme I: Born Cycle for the Binding of Oxidized, One-Electron-Reduced and Two-Electron-Reduced FAD to the Apoenzyme of Salicylate Hydroxylase and for the Binding of Salicylate to the Oxidized, One-Electron-Reduced and Two-Electron-Reduced Forms of the Holoenzyme[a]



[a] Potential values for the free and the salicylate-bound enzyme were obtained at pH 7.6, 10 °C. [b] Wang et al. (1984), pH 7.0, 6 °C. [c] Wang and Tu (1984), pH 7.6, 23 °C. [d] Draper and Ingraham (1968), pH 7.6, 20 °C.

of reducing the inhibited enzyme. The absence of semiquinone indicates that the potential value for the second electron is much more positive than the potential value for the first electron. The potential for the first electron is now shifted negatively, closer to the potential for the NADH/NAD$^+$ couple. The negative shift of the first electron suggests that chloride is more tightly bound to the oxidized form of the enzyme than to the one-electron-reduced and two-electron-reduced forms of the enzyme.

Discussion

As in any complexation system, the ratio of binding constants of the electroactive species (here, flavin) reflects the redox potentials of those species. The redox potentials at pH 7.6 and the previously measured binding constants for oxidized flavin (Fl_{ox}) to apoprotein (E) and salicylate hydroxylase enable the calculation of the conditional binding constants of flavin to apoprotein (Scheme I). Salicylate binding causes the binding constants of Fl_{ox}, $Fl^{\cdot-}$, and $Fl_{red}H^-$ to apoprotein to shift from 6.7×10^7, 7×10^9, and 4.9×10^{12} M^{-1} to 5.4×10^{12}, 4×10^{14}, and 7.4×10^{16} M^{-1}, respectively. The binding of Fl_{ox}, $Fl^{\cdot-}$, and $Fl_{red}H^-$ to apoprotein in the presence of salicylate is increased by a factor of 10^4–10^5. It is also clear that salicylate is bound almost to the same extent to all three oxidation states of the enzyme with binding constants of 8.0×10^4 M^{-1} to the oxidized, 5×10^4 M^{-1} to the one-electron-reduced, and 1.5×10^4 M^{-1} to the two-electron-reduced enzyme. The data presented here show that the potential values for the individual electrons are shifted positive of those of FAD for both free enzyme and substrate-bound enzyme. The potential of the second electron for both free and substrate-bound enzyme is shifted more than the potential of the first electron. These facts are consistent with extensive hydrogen bonding to the flavin in all three oxidation states with increasing hydrogen-bonding interactions to the one-electron- and two-electron-reduced flavin for both free and substrate-bound enzyme. For *p*-hydroxybenzoate hydroxylase, the most extensively studied FAD-dependent hydroxylase, extensive hydrogen bonding to the flavin for the oxidized enzyme has been shown by X-ray structure (Hofsteenge et al., 1980) and resonance Raman studies (Bienstock et al., 1986).

The −0.020-V potential shift obtained upon salicylate binding to salicylate hydroxylase clearly demonstrates that the enzyme is designed to keep the substrate tightly bound to the oxidized and both reduced forms of the enzyme (Scheme I). The potential shift is not in a direction that would enhance the rate of reduction by NADH. It appears from the thermodynamics that the binding energy of the substrate stored in the protein is not transferred to the flavin and that the binding of substrate causes little perturbation of the flavin environment. However, different spectral properties of the oxidized enzyme and a change in protonation state of the flavin semiquinone are observed upon substrate binding. Similar observations have been made for *p*-hydroxybenzoate hydroxylase in which spectral changes (Howell et al., 1972) and conformational changes (Wierenga et al., 1979) are observed on substrate binding. However, resonance Raman spectroscopy, which exclusively responds to the flavin environment, shows that minimal change in the flavin environment occurs when substrate binds to the oxidized enzyme (Bienstock et al., 1986).

The protonation state of the flavin semiquinone did change on salicylate binding, indicating that in the presence of the substrate a proton is transferred with the first electron. These observations are consistent with hydrogen bonding at the N-1 position of the flavin for the free enzyme. The neutral semiquinone could be stabilized with additional hydrogen bonding at N-5 in the reduced enzyme upon substrate binding (Mizzer et al., 1981) and still cause minimal perturbation of the flavin environment. Changes in the protonation state of a flavin semiquinone upon ligand binding have previously been observed. Binding of crotonyl-CoA to general acyl-CoA dehydrogenase caused the protonation state of the flavin semiquinone to change from neutral to anionic (Mizzer & Thorpe, 1981). Similar observations were made upon product binding to glucose oxidase (Massey et al., 1966). The neutral semiquinone of D-amino-acid oxidase has been observed (Yagi et al., 1972) upon anaerobic addition of excess benzoate to the photochemically generated anionic semiquinone of the free enzyme.

The molar absorptivity obtained for the neutral semiquinone at 570 nm, 6600 M^{-1} cm^{-1}, is only 12–18% higher than molar absorptivities that have been reported for shethna flavoprotein, 5600 M^{-1} cm^{-1} (Barman & Tollin, 1972), and for general acyl-CoA dehydrogenase, 5900 M^{-1} cm^{-1} (Thorpe et al., 1979; Gustafson et al., 1986). This way of estimating the molar absorptivity has inherent in it 5–10% error as mentioned earlier. There is a possibility that the equilibrium position shifts upon freezing (Porras & Palmer, 1982); however, this is unlikely since salicylate hydroxylase has only one redox-active center. If freezing were to change the amount of semiquinone formed, it would assume fast protein–protein interaction that is unlikely on this time scale. In agreement with this, Massey and Palmer (1966) quantitated flavin semiquinones at 200 K and room temperature and found that the results obtained at the two different temperatures were in excellent agreement. Assuming the well-established molar absorptivity for *Megasphaera elsdenii* flavodoxin of 4500 M^{-1} cm^{-1} at 580 nm (Mayhew & Massey, 1968), the amount of semiquinone thermodynamically stabilized in potentiometric

titrations of the substrate-bound enzyme increased 2–7% depending on pH. The effect of this is to decrease the potential separation of the individual electrons; however, these calculations do not change the potential/pH dependence of the electrons. The binding constant calculated for the one-electron-reduced holoenzyme and salicylate in Scheme I is enhanced, changing from 5×10^4 to 8×10^4 M^{-1}, still indicating strong binding of salicylate to all three oxidation states of the enzyme.

In contrast to the behavior of substrate-bound enzyme, it was observed that when the effector, benzoate, is bound to the enzyme, the midpoint potential shifts even more negative. From the shifts in midpoint potential the dissociation constant of the two-electron-reduced enzyme was calculated to be ~0.6 M. From the ratio of dissociation constants a maximum shift of −0.07 V would be expected when all three forms of the enzyme are saturated. At a benzoate concentration of 0.035 M, the anionic semiquinone was observed. This indicates that some of the one-electron-reduced form of the enzyme is not bound to benzoate and that the benzoate is bound less tightly to the one-electron-reduced form of the enzyme than to the oxidized form. At a benzoate concentration of 0.2 M no flavin semiquinone could be detected, indicating that the one-electron-reduced form is completely complexed with benzoate. Therefore, it is concluded that the benzoate binding to the one-electron-reduced form of the enzyme is tighter than the binding to the two-electron-reduced form but not as tight as the binding to the oxidized form of salicylate hydroxylase. It is possible that the effector dissociates from the enzyme–effector complex upon reduction, leaving the two-electron-reduced enzyme in a noncatalytic state that then can react with free oxygen in solution to form H_2O_2.

With the free enzyme a kinetic stabilization of the anionic semiquinone was observed at pH values between 6.8 and 8.0 in dithionite and coulometric reductions. Only thermodynamic stabilization of any form of semiquinone was seen when enzyme was complexed with salicylate or the effector, benzoate (at 0.2 M concentration), in dithionite and coulometric titrations. This suggests that the transfer of the second electron for the free enzyme is kinetically slow and is more difficult to transfer even though it has much more positive reduction potential. This effect is independent of the charge of the reducing agent (dithionite is negatively charged, while methylviologen is positively charged). An alternative explanation is that the proton transfer is rate limiting in the free enzyme.

It is concluded that the relatively slow reduction rate of the free enzyme, compared to those of the substrate- and effector-bound enzyme, is caused by the kinetically slow transfer of the second electron or the proton in the free enzyme both with NADH (two-electron donor) and with dithionite and methylviologen (one-electron donors). The formation of the neutral semiquinone in the presence of salicylate indicates that only in its presence is proton access permitted. Since the physiological reducing agent NADH may be taken as a synchronous two-electron donor, proton transfer must be synchronous with electron transfer, and the slow reduction in the absence of substrate could be caused by unavailability of the proton in the transition state. In order for a molecule to act as an effector, it has to remain bound to the one-electron-reduced form of the enzyme and possibly keep the flavin in a configuration in both the oxidized and one-electron-reduced states that allow for fast electron transfer of the second electron or fast transfer of the proton. However, for a molecule to act as substrate it has to remain bound to the two-electron-reduced enzyme. The continued binding of the substrate near the N-5 position of the flavin in the two-electron-reduced enzyme is important because then the substrate can be hydroxylated. The calculated ratio for conditional binding constants for salicylate to oxidized and reduced enzyme is 5, with the salicylate being more tightly bound to the oxidized form of the enzyme. However, the ratio of binding constants for benzoate binding to the oxidized and reduced enzyme is ~250, with benzoate being much more tightly bound to the oxidized enzyme.

For salicylate hydroxylase there are pseudosubstrates (partially hydroxylated) that have substrate activity between salicylate (best substrate) and benzoate (best nonsubstrate effector). Those pseudosubstrates enhance the rate of reduction by NADH when bound to the enzyme compared to the reduction of free enzyme by NADH, and their K_m values are between that of salicylate and benzoate (White-Stevens & Kamin, 1972). One possible explanation suggested by the dissociation of benzoate from the reduced form of the enzyme is that the path taken by the oxygenated intermediate may be determined by how tightly the aromatic pseudosubstrate is bound to the reduced enzyme.

The data presented here are consistent with what is known about salicylate hydroxylase and other flavin hydroxylases. A negative potential shift of −0.020 V upon substrate binding agrees with binding constants measured for the *P. putida* (Takemori et al., 1972) and the *P.* sp. ATCC 29351 (Presswood & Kamin, 1976) salicylate hydroxylases, where −0.020- and −0.036-V potential shifts would have been expected, respectively. A small positive potential shift is expected for melilotate hydroxylase (Schopfer & Massey, 1980) on the basis of the binding constants for the substrate, 2-hydroxycinnamate, to the reduced and oxidized enzyme. Likewise, small potential shifts are expected for *p*-hydroxybenzoate hydroxylase on the basis of the binding constants for *p*-hydroxybenzoate to the oxidized (Howell et al., 1972) and reduced (Entsch et al., 1976) enzyme. The relatively tight binding of substrate to both oxidized and reduced forms of the enzyme fits with the known catalytic mechanism of the hydroxylases.

In conclusion it is clear that the substrate binding does not make the reduction of the flavin thermodynamically more favorable. This mode of thermodynamic activation by substrate can clearly be ruled out as contributing to the 290-fold rate enhancement. Thus, the alternative explanation becomes more plausible; i.e., the conformational change on substrate binding causes the reorientation of NADH with respect to the flavin to give more favorable geometry for proton and/or electron transfer. The change in protonation state of the semiquinone stabilized and the absence of kinetic stabilization of the semiquinone also support a conformational change upon substrate binding.

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Registry No. Benz, 65-85-0; Sal, 69-72-7; Sal hydroxylase, 9059-28-3; chloride, 16887-00-6.

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Inactivation of γ-Aminobutyric Acid Aminotransferase by (*Z*)-4-Amino-2-fluorobut-2-enoic Acid†

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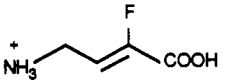
ABSTRACT: (*Z*)-4-Amino-2-fluorobut-2-enoic acid (**1**) is shown to be a mechanism-based inactivator of pig brain γ-aminobutyric acid aminotransferase. Approximately 750 inactivator molecules are consumed prior to complete enzyme inactivation. Concurrent with enzyme inactivation is the release of 708 ± 79 fluoride ions; transamination occurs 737 ± 15 times per inactivation event. Inactivation of [^{3}H]pyridoxal 5′-phosphate ([^{3}H]PLP) reconstituted GABA aminotransferase by **1** followed by denaturation releases [^{3}H]PMP with no radioactivity remaining attached to the protein. A similar experiment carried out with 4-amino-5-fluoropent-2-enoic acid [Silverman, R. B., Invergo, B. J., & Mathew, J. (1986) *J. Med. Chem. 29*, 1840–1846] as the inactivator produces no [^{3}H]PMP; rather, another radioactive species is released. These results support an inactivation mechanism for **1** that involves normal catalytic isomerization followed by active site nucleophilic attack on the activated Michael acceptor. A general hypothesis for predicting the inactivation mechanism (Michael addition vs enamine addition) of GABA aminotransferase inactivators is proposed.

Allan et al. (1979) briefly noted in an abstract that 4-amino-2-fluorobut-2-enoic acid (**1**) was a time-dependent in-



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activator of the pyridoxal 5′-phosphate dependent enzyme γ-aminobutyric acid (GABA)[1] aminotransferase; no inacti-

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