Protocatechuate 3,4-Dioxygenase: Implications of Ionization Effects on Binding and Dissociation of Halohydroxybenzoates and on Catalytic Turnover†

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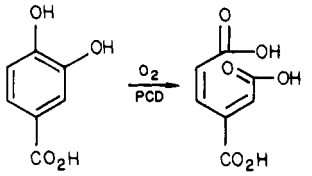
ABSTRACT: The effect of pH upon the binding and dissociation of halohydroxybenzoate inhibitors of protocatechuate 3,4-dioxygenase (PCD) and on catalytic turnover with the substrates protocatechuic acid (PCA) and 3,4-dihydroxyphenylpropionic acid (DHPP) has been investigated. The k_{cat} profile for PCA shows a simple sigmoidal pH dependence, with an apparent pK_a of 7.0 ± 0.1. The k_{cat}/K_m profile obtained in phosphate exhibits an apparent pK_a of ~7, while that obtained in zwitterionic buffers shows only slight variation in the pH region from 5.5 to 7.5 and a decrease above pH 8. The pH dependence of breakdown of the E·S·O_2 complex, formed from the slow substrate, DHPP, also exhibits a sigmoidal variation with pH, with an apparent pK_a of 8.0 ± 0.1. The pH dependence of binding of the potent inhibitors 3-fluoro-4-hydroxybenzoic acid and 3-chloro-4-hydroxybenzoic acid demonstrates that the strong binding of these compounds is not attributable to the increased acidity of the phenolic moiety. A linear free-energy plot of the 3-halo-4-hydroxybenzoates reveals that steric factors are important in determining the relative potency of these inhibitors. To obtain further information on the interaction of PCD with the inhibitors, we measured the dissociation rates via stopped-flow displacement. These dissociation rates show a dramatic increase at alkaline pH values, which parallels the rise in both K_I and K_m. In contrast, the pH dependence of 4-nitrocatechol (4-NC) dissociation is bell-shaped and decreases at alkaline pH. The kinetics of association of 4-NC with PCD were analyzed and are consistent with a rapid ligation step followed by a slow process which yields the characteristic 550-nm peak of the PCD·4-NC complex. With regard to catalytic turnover, evidence has been obtained that the k_{cat} profile reflects an ionization which directly facilitates breakdown of the E·S·O_2 complex. The implications of these findings are discussed in terms of the structure of the E·S·O_2 complex and the mechanism of PCD catalysis.

Dioxygenases are a widespread class of enzymes which play a critical role in the degradation of aromatic compounds. The mechanism by which dioxygenases are able to catalyze the oxygenolytic cleavage of aromatic rings has recently become a subject of considerable interest. One of these enzymes, protocatechuate 3,4-dioxygenase (PCD),[1] which catalyzes the intradiol cleavage of protocatechuic acid (PCA) as shown in eq 1, has been identified in a number of microbial genera



(1)

including *Pseudomonas* (Stanier & Ingraham, 1954), *Thiobacillus* (Wells, 1972), and *Acinetobacter* (Hou et al., 1976), but the crystalline enzyme obtained from *p*-hydroxybenzoate-induced *Pseudomonas aeruginosa* is particularly suitable for study, due to its high stability and relative ease of isolation (Fujisawa & Hayaishi, 1968). This enzyme has a molecular weight of 700 000 and is composed of eight identical subunits, each of which contains one essential ferric iron atom as the sole cofactor (Fujisawa et al., 1972a; Tyson, 1975, May et al., 1978).

The native enzyme exhibits a broad visible absorption maximum at ~450 nm, which is shifted to ~480 nm upon anaerobic addition of substrates and further altered to ~500 nm upon introduction of O_2 (Fujisawa et al., 1972a). Similarly, the intensity of the EPR signal of the native enzyme at $g = 4.3$ is drastically reduced by substrate addition (Fujisawa et al., 1972a), and EPR and Mossbauer studies (Que et al., 1976, 1977) have established that the iron remains in the high-spin ferric state throughout the catalytic cycle. Recently, resonance Raman spectra of native PCD (Felton et al., 1978; Keyes et al., 1978; Tatsuno et al., 1978) have identified tyrosinate ligation, and on the basis of the Raman spectra of complexes of inhibitory halohydroxybenzoates and substrate, we have proposed phenolate ligation and chelation, respectively, of the active-site iron by these compounds, with no loss of tyrosine ligands (Felton et al., 1978).

The kinetics of the PCD reaction have been firmly established by stopped-flow (Fujisawa et al., 1971, 1972b) as well as steady-state kinetics (Hori et al., 1973) to obey a compulsory ordered bi–uni mechanism, in which the organic substrate binds prior to O_2. Since the substrate initially contains two ionizable phenolic protons which in the product are converted to carboxylic acid groups with a large decrease in pK_a, it seems likely that the loss of these protons is intimately associated with binding and/or catalysis. We have now carried out a study of the pH dependence of the binding of nonme-

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[1] Abbreviations used: PCD, protocatechuate 3,4-dioxygenase [protocatechuate:oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.3]; 4-NC, 4-nitrocatechol; 3-FHB, 3-fluoro-4-hydroxybenzoic acid; 4-FHB, 4-fluoro-3-hydroxybenzoic acid; 4-HB, 4-hydroxybenzoic acid; DHPP, 3,4-dihydroxyphenylpropionic acid; 3,5-diClHB, 3,5-dichloro-4-hydroxybenzoic acid; PCA, protocatechuic acid; PCL, protocatechualdehyde; 3-ClHB, 3-chloro-4-hydroxybenzoic acid; 3-BrHB, 3-bromo-4-hydroxybenzoic acid.

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tabolized hydroxybenzoate inhibitors as well as of the catalytic turnover process with both the normal substrate PCA and the slowly reacting substrate homologue 3,4-dihydroxyphenylpropionic acid (DHPP). The results obtained in this study are presented herein and discussed in terms of the dioxygenase reaction mechanism.

Materials and Methods

All commercial materials were of the highest grade obtainable. 3-Fluoro-4-hydroxybenzoic acid (3-FHB) was prepared from *o*-fluoroanisole as previously described (May et al., 1978). 3-Chloro-4-hydroxybenzoic acid hemihydrate (3-ClHB), 3,5-dichloro-4-hydroxybenzoic acid, and 4-nitrocatechol (4-NC) were obtained from Aldrich, recrystallized from distilled water, and dried in vacuo prior to use. 3-Bromo-4-hydroxybenzoic acid hemihydrate (3-BrHB) was synthesized from 4-hydroxybenzoic acid by treatment with 1 equiv of bromine in acetic acid. The reaction mixture was poured into ice water, after standing overnight, and the precipitated crude product was collected. A further crystallization from acetic acid–water (2:1) was necessary to remove a small amount of contaminating 3,5-dibromo-4-hydroxybenzoic acid and yielded colorless needles, mp 164–165 °C [lit. mp 163.8–165 °C (Hussey & Wilk, 1950)[2]]. Anal. Calcd for $C_7H_5BrO_3 \cdot 0.5H_2O$: C, 37.20; H, 2.68. Found: C, 37.15; H, 2.56. Protocatechuic acid and protocatechualdehyde, from various commerical sources, were decolorized with charcoal and recrystallized from water before use. 3,4-Dihydroxyphenylpropionic acid (DHPP), obtained from Aldrich, was dissolved in ethyl acetate, decolorized by treatment with charcoal, and crystallized by addition of ice-cold chloroform. *N*,*N*-Bis(2-hydroxyethyl)glycine (Bicine) was from Eastman, and 3-(*N*-morpholino)propanesulfonic acid (Mops) and 2-(*N*-morpholino)ethanesulfonic acid (Mes) were obtained from Sigma.

Growth of *P. aeruginosa* and isolation of PCD were performed as previously described (May et al., 1978). Visible and ultraviolet absorption spectra were obtained by using either a Beckman Acta MVI or an Aminco DW-2 spectrophotometer. Enzyme activity was measured spectrophotometrically following the decrease in absorbance at 290 nm (Stanier & Ingraham, 1954). Protein concentrations were determined by using a dye-binding assay (Bradford, 1976), with a calibration curve prepared from purified PCD, or, for highly purified samples, by using $E_{280}^{1\%} = 13.2$ (Fujisawa & Hayaishi, 1968) and assuming a molecular weight of 700 000.

The steady-state kinetic experiments were performed on the Aminco DW-2 spectrophotometer equipped with an Aminco-Morrow stopped-flow accessory. The reservoir syringes, mixing chamber, and observation chamber were maintained at 25 °C with a Lauda K-2/RD circulating water bath. Buffer solutions were air-saturated and adjusted to an ionic strength of 0.1 with NaCl; typical $[E]_0 = 8.6 \times 10^{-9}$ M (active sites) and $[S]_0 = 10$–200 μM. The reaction was followed in the dual-wavelength mode, with the sample monochromator set on 290 nm and the reference monochromator at 270 nm. From four to six reaction traces at each [S] were obtained, and the mean of the initial rates was determined. K_m, k_{cat}, and k_{cat}/K_m values were obtained by using a computer program to fit the hyperbolic form of the Michaelis–Menten equation (Cleland, 1967).

[2] There is some confusion in the literature as to the melting point of 3-BrHB; values reported range from 148 to 177 °C. This has been attributed to different hydrated forms and/or polymorphism (Brink, 1966).

The advantages of the dual-wavelength stopped-flow technique over conventional spectrophotometric techniques are the following: (1) a full kinetic series requires much less material and time; (2) the combined $\Delta\epsilon$ at 270 and 290 nm is about twice that at either wavelength alone, thus increasing sensitivity and therefore precision in the data, especially at low substrate concentrations; (3) the reaction trace contains the entire course of the reaction, thereby facilitating accurate initial rate determination; (4) the variation of the combined $\Delta\epsilon$ with pH is much less pronounced than that of either single wavelength, so that this technique is ideal for a study of the pH dependence of the steady-state kinetic properties of PCD. For the K_I measurements, both inhibited and uninhibited enzyme solutions were prepared, each was mixed with several protocatechuic acid solutions of varying concentration, and the reaction was followed as above. All the 4-hydroxybenzoates examined show simple competitive inhibition patterns in double-reciprocal plots, and K_I values were estimated from the ratio of slopes of the inhibited and uninhibited series.

The turnover rates with the slow substrate, 3,4-dihydroxyphenylpropionic acid, were obtained by using conventional spectrophotometric techniques (Fujisawa et al., 1972b). Enzyme solutions were brought to ~1 mM in substrate by addition of 0.1–0.2 mg of the solid material, followed by rapid gentle mixing to dissolve the solid. Because of the large amount of acid produced during the reaction, careful pH control was essential.

The displacement experiments were performed at 25 °C in the stopped-flow spectrophotometer. For halohydroxybenzoates, enzyme–inhibitor complexes were prepared by using a concentration sufficient to ensure saturation, based on the K_I values obtained in the steady-state kinetic measurements. The enzyme–inhibitor complexes were then mixed with protocatechualdehyde solutions (1–5 mM), and the reaction was followed at 560 nm, with a reference wavelength of 750 nm. For 4-NC displacement reactions, solutions of PCD (0.5–1.0 mg/mL; ~10 μM in active sites) were prepared in the appropriate buffers and precomplexed by addition of excess 4-NC (20 μM). The PCD·4-NC complex was then mixed with a large excess of either 3-FHB or 3-ClHB (2–5 mM) in the stopped-flow spectrophotometer and the displacement followed at 550 nm, with a 430-nm reference wavelength, as reported previously (May et al., 1978). Simple first-order kinetics were observed in all cases. For the association reactions, PCD solutions prepared as above were mixed with excess 4-NC (100–500 μM) and the absorbance changes at either 430 or 550 nm followed with reference wavelength of 700 nm. The reaction traces were stored on a Tektronix 564B storage oscilloscope and recorded with a Polaroid camera. The first-order rate constants were then obtained from logarithmic plots of the reaction trace.

Results

Inhibition by Halohydroxybenzoates. Halo-substituted hydroxybenzoates are potent inhibitors of PCD. We have previously reported in detail on the isomeric fluorinated analogues 3-FHB and 4-FHB, the former being the most potent PCD inhibitor known and an excellent titrant and spectral probe for both absorption and Raman spectroscopy (May et al., 1978; Felton et al., 1978). We have also shown that the chlorinated analogue 3-ClHB is a potent inhibitor and have used this compound extensively in our Raman studies (Felton et al., 1978). Table I presents a summary of K_I data for various halohydroxybenzoates, all of which are simple competitive inhibitors of PCD, along with the various pK_a values for these compounds. Although the E·I complexes of

Table I: K_I and pK_a Values for Hydroxybenzoates and Halohydroxybenzoates

compd	K_I (μM)[a]	phenol pK_a
4-hydroxybenzoate	100[b]	9.3[c]
3-fluoro-4-hydroxybenzoate	0.5[b]	7.8[d]
3-chloro-4-hydroxybenzoate	3.2	7.5[d]
3-bromo-4-hydroxybenzoate	18	7.5[d]
3,5-dichloro-4-hydroxybenzoate	44	5.7[d]
3-hydroxybenzoate	>1000	9.9[c]
4-fluoro-3-hydroxybenzoate	300	8.5[d]

[a] At pH 7.5. [b] From May et al. (1978). [c] From *CRC Handbook of Chemistry and Physics*. [d] Values obtained from spectrophotometric titration data.

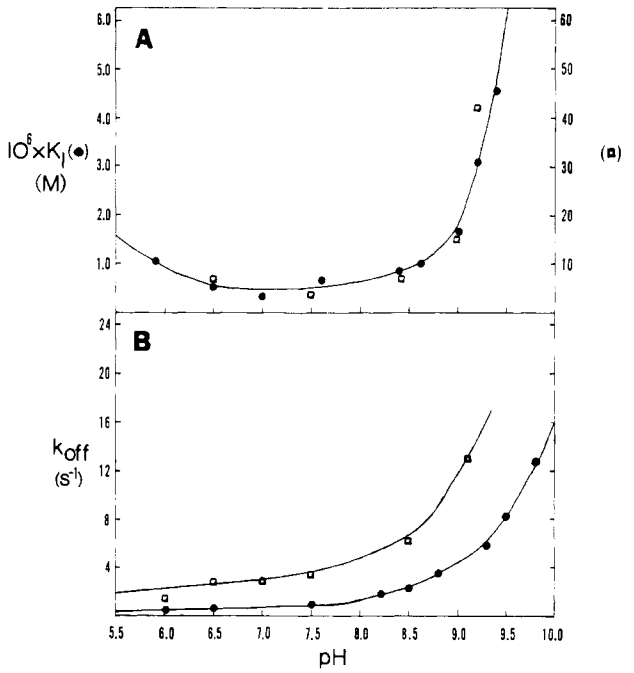


FIGURE 1: pH dependence of K_I and k_{off} for 3-halo-4-hydroxybenzoates. (A) pH dependence of K_I for 3-fluoro-4-hydroxybenzoic acid (●) and 3-chloro-4-hydroxybenzoic acid (□). K_I values were estimated from the slope ratios of double-reciprocal plots of steady-state kinetic data obtained as described under Materials and Methods. Values for 3-FHB are plotted on the left-hand ordinate scale, while values for 3-ClHB are plotted on the scale of the right-hand ordinate. (B) pH dependence of displacement of 3-fluoro-4-hydroxybenzoic acid (●) and 3-chloro-4-hydroxybenzoic acid (□). Dissociation of the bound inhibitors was measured by displacement with protocatechualdehyde as described in the text under Materials and Methods.

all inhibitors containing 4-OH groups exhibit virtually identical spectral features [see May et al. (1978)], the much increased inhibitor potency caused by 3-halo substitution clearly does not correlate simply with increased acidity of the *p*-hydroxyl. Moreover, as shown in Figure 1, the pH dependence of K_I for both 3-FHB and 3-ClHB does not reflect the pK_a of ~7.5 for the free inhibitors. We note that although the inhibitory potencies of 3-FHB and 3-ClHB differ by about an order of magnitude, the K_I vs. pH profiles for these two compounds are virtually superimposable. Thus, *simple* prior ionization of the free inhibitors is not responsible for their increased potency, despite the fact that our Raman results clearly demonstrate Fe–O ligation in the E·I complexes of both 3-FHB and 3-ClHB. The K_I values for fluoro, chloro, and bromo substitutions correlate well with the steric substituent constants (Taft, 1953, 1963). The slope of this plot, δ, provides a measure of the sensitivity of the system to steric effects (Taft, 1963), and we find in this case a value of 3.1, indicating very high sensitivity to the steric requirements of substituents at

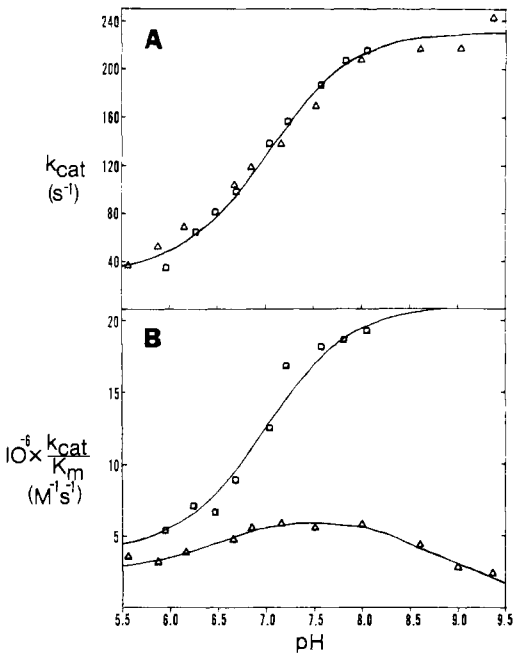


FIGURE 2: pH dependence of the steady-state kinetic parameters k_{cat} and k_{cat}/K_m. (A) pH dependence of k_{cat}. k_{cat} values were determined as described under Materials and Methods. All measurements were made in either 0.05 M sodium phosphate (□) or 0.05 M Mops, Mes, or Bicine (△), with the ionic strength adjusted to 0.1 with NaCl and the instrument thermostated to 25 ± 0.1 °C. The curve shown is that calculated for a simple ionization with pK_a = 7.0 ± 0.1, assuming limiting rates of 30 s^{-1} at acidic pH and 230 s^{-1} at alkaline pH. k_{cat} values are reported per active site, assuming eight sites per M_r 700 000. (B) pH dependence of k_{cat}/K_m. k_{cat}/K_m values were determined as described under Materials and Methods, and conditions were identical with those described above for the k_{cat} measurements. For the data obtained in phosphate buffers (□), the curve is that calculated for a simple ionization with a pK_a of 7.0 ± 0.1, assuming limiting values of 4.0 × 10^6 M^{-1} s^{-1} at acidic pH and 2.1 × 10^7 M^{-1} s^{-1} at alkaline pH. For the data obtained in Mops, Mes, or Bicine (△), the curve shown is hand drawn for the purpose of comparison.

position 3. In line with this correlation, the K_I of 3,5-diClHB is increased by an order of magnitude over the monochloro compound. In contrast, we have found no significant difference in the inhibitory potency of 4-hydroxybenzoate and 2,4-dihydroxybenzoate (R. S. Phillips, unpublished experiments).

pH Dependencies of Catalytic Parameters. Although binding of hydroxybenzoate inhibitors is apparently independent of their ionization state in free solution, the catalytic parameters for substrate oxygenation do exhibit pH dependencies. In these experiments, kinetic parameters were obtained via the dual-wavelength stopped-flow technique described under Materials and Methods, which has several operational advantages, plus increased sensitivity, especially at low substrate concentrations.

As shown in Figure 2, the pH dependence of k_{cat} fits a simple sigmoidal titration curve, with an apparent pK_a of 7 ± 0.1. Over the entire pH range, simple Michaelis–Menten kinetics were observed, and the steady-state parameters were computer calculated by using the program of Cleland (1967). On the basis of steady-state kinetic analyses and stopped-flow studies, Fujisawa et al. (1972b) have established that PCD catalysis follows a compulsory ordered bi–uni mechanism with the organic substrate binding first (eq 2). At pH 7.5, Fujisawa

$$\mathrm{PCD} \underset{k_2}{\overset{k_1,\,S}{\rightleftharpoons}} \mathrm{PCD{\cdot}S} \underset{k_4}{\overset{k_3,\,O_2}{\rightleftharpoons}} \mathrm{PCD{\cdot}S{\cdot}O_2} \xrightarrow{k_5} \mathrm{PCD} + \mathrm{P} \qquad (2)$$

et al. (1971) also established that k_{cat} evaluated from steady-state turnover was identical with the rate of "E·S·O_2"

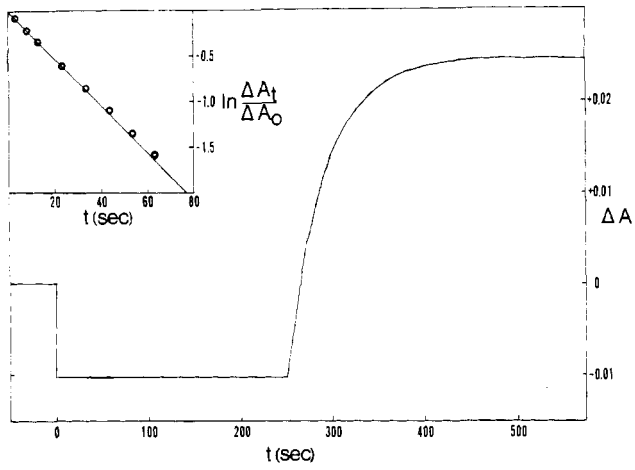


FIGURE 3: Direct observation of turnover with 3,4-dihydroxyphenylpropionic acid. Turnover with the slowly reacting substrate, 3,4-dihydroxyphenylpropionic acid, was followed as described under Materials and Methods. 1 mL of PCD solution (1 mg/mL; 11 μM) in 0.05 M Bicine buffer, pH 7.94, was placed in the spectrophotometer. Addition of excess solid substrate (0.1 mg; $\sim$1 mM) at $t = 0$ causes an instantaneous decrease in absorbance at 450 nm. When the O_2 is depleted, an increase in absorbance is observed. The first-order plot (inset) shows that turnover follows a simple first-order process, with a rate constant of 0.0255 s^{-1}.

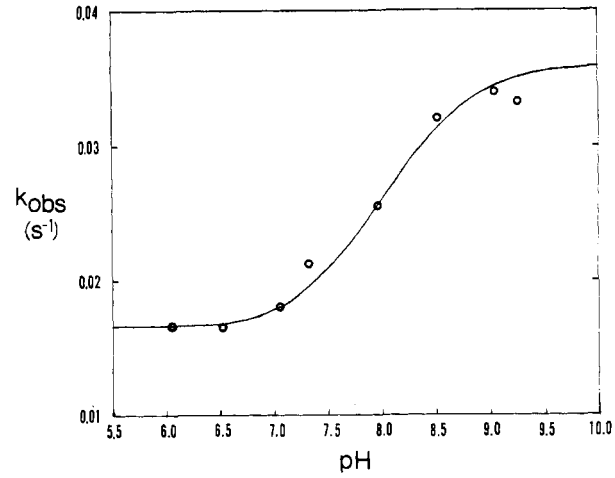


FIGURE 4: pH dependence of E·S·O_2 breakdown for 3,4-dihydroxyphenylpropionic acid. Rates were determined as described under Materials and Methods, in experiments similar to that illustrated in Figure 3. The curve shown is the theoretical curve for a simple ionization calculated in the same manner as in Figure 3, with an apparent pK_a of 8.0 $\pm$ 0.1, assuming limiting rates of 0.016 s^{-1} at acidic pH and 0.036 s^{-1} at alkaline pH.

decomposition (k_5). Thus, if no change in rate-determining step occurs over the pH range of our studies, the pK_a at 7 reflected in our k_{cat} profile can be interpreted as reflecting an ionization affecting breakdown of the ternary E·S·O_2 complex. Further support for this conclusion was obtained in the following experiments with the slow substrate, DHPP.

DHPP reacts with PCD via the mechanism of eq 2, but the value of k_5 is several orders of magnitude lower than that for protocatechuic acid (Fujisawa et al., 1972b). Thus, one can quantitatively form the E·S·O_2 complex under steady-state conditions and follow its breakdown by using conventional spectrophotometric techniques, allowing direct observation of the elementary step k_5. Typical data obtained in these experiments are illustrated in Figure 3 [compare Fujisawa et al. (1972b)]. Upon addition of excess DHPP, a rapid decrease in absorbance is noted, corresponding to formation of the E·S·O_2 complex. Upon depletion of oxygen, in $\sim$5 min, a smooth first-order reaction is observed, as the absorbance increases to that characteristic of the anaerobic E·S complex. The reaction was followed either at 450 nm or at 700 nm above pH 9. As shown in Figure 4, the pH dependence of k_5 for DHPP is sigmoidal, analogous to the pH dependence of k_{cat} for PCA, thus strongly supporting our conclusion that both profiles reflect an ionization affecting breakdown of the E·S·O_2 complex. We note that the change in substrate structure from PCA to DHPP results in a pK_a shift of $\sim$1 unit, but in both cases ionization facilitates catalysis.

Also shown in Figure 2B are k_{cat}/K_m profiles, and a drastic buffer effect is apparent which is not reflected in the k_{cat} profile. The k_{cat}/K_m data in phosphate fit well to a sigmoidal curve, with a pK_a of $\sim$7, mirroring the k_{cat} data, but in zwitterionic buffers the k_{cat}/K_m data are relatively invariant with pH. For the mechanism of eq 2, if [O_2] remains much larger than its K_m and if the steady state is maintained over the entire pH range, then $k_{cat} = k_5$ and $k_{cat}/K_m = k_1$. As stated above, the direct measurements of Fujisawa et al. (1971) at pH 7.5 and our turnover experiments with DHPP support the conclusion that the k_{cat} profile in Figure 2A indeed reflects simply an ionization effect on k_5 and thus imply that these kinetic conditions indeed hold. If so, a virtually flat k_{cat}/K_m profile, such as that in Figure 2B for the zwitterionic buffers, implies no ionization effects on substrate binding. This is in excellent accord with our results with the halohydroxybenzoate inhibitors, where the only pH effect observed on K_I is a rapid rise above pH 8.5, paralleled by an increase in k_{off} for these inhibitors (see below). The exact nature of the phosphate effect, which obviously affects only k_{cat}/K_m and not k_{cat}, is not clear. If in phosphate, above pH $\sim$6.5, the condition [O_2] $\gg$ $K_m^{O_2}$ no longer holds, then k_{cat}/K_m is given by

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_3 k_5 [O_2]}{k_2(k_4 + k_5) + k_3 k_5 [O_2]} \qquad (3)$$

and the profile can be reflecting complicated combinations of pH dependencies. However, if this is so, then the apparent k_{cat} in phosphate is also a complex function.

Displacement Studies with 4-Hydroxybenzoates. In order to obtain more information on the interaction of the 3-halo-4-hydroxybenzoates with PCD, we used the displacement technique of Gutfreund (1972) to examine the kinetics of dissociation of the PCD·inhibitor complexes. We have previously used the same technique to demonstrate that the dissociation of 4-nitrocatechol (4-NC) from PCD is a slow process (May et al., 1978). For the interaction of PCD with two simple competitive inhibitors, I_1 and I_2, if a solution of PCD·I_1 complex is flowed against a sufficient excess of I_2, a simple first-order reaction will be observed when $k_1 \ll k_{+2}[I_2]$ and $k_{+1}[I_1] \ll k_{+2}[I_2]$. Thus, the dissociation rate constants for both 3-FHB and 3-ClHB were obtained by using the potent inhibitor protocatechualdehyde (PCL) as the displacing ligand. As was the case in our previously reported 4-NC displacement, the kinetic situation in these experiments follows the simple scheme of eq 4, as evidenced by the following observations:

$$\text{PCD·I}_1 \underset{k_{+1}}{\overset{k_{-1}}{\rightleftharpoons}} \text{PCD} + \text{I}_1 + \text{I}_2 \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{PCD·I}_2 \qquad (4)$$

(a) simple first-order kinetics are observed in the stopped-flow spectrophotometer (Figure 5); (b) as required by the kinetic scheme, identical dissociation rate constants were obtained when the concentration of PCL was varied from 0.5 to 5.0 mM; (c) upon omission of the hydroxybenzoate inhibitors, no reaction is observed at these wavelengths, the association of PCL with uncomplexed PCD being very rapid; (d) equilibrium displacement experiments under the conditions used in the

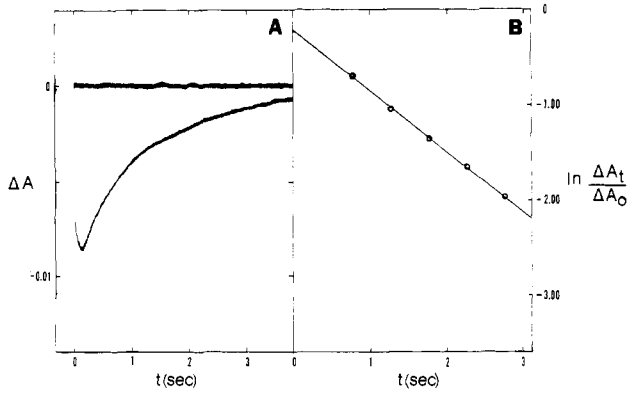


FIGURE 5: 3-Fluoro-4-hydroxybenzoate displacement from PCD. (A) Reaction trace of typical displacement run as described under Materials and Methods. Native PCD (1.3 mg/mL; 15 μM active sites), to which 39.8 μM 3-FHB was added, was mixed with 5 mM PCL in the stopped-flow spectrophotometer and the reaction followed at 560 and 750 nm. The buffer used was 0.05 M sodium phosphate, pH 6.5, and the temperature was 25 °C. The instrumental response time in this experiment was 10 ms. (B) Semilog plot of the data shown in (A). The rate, obtained from the slope of the plot by least-squares analysis, is 0.60 s^{-1}.

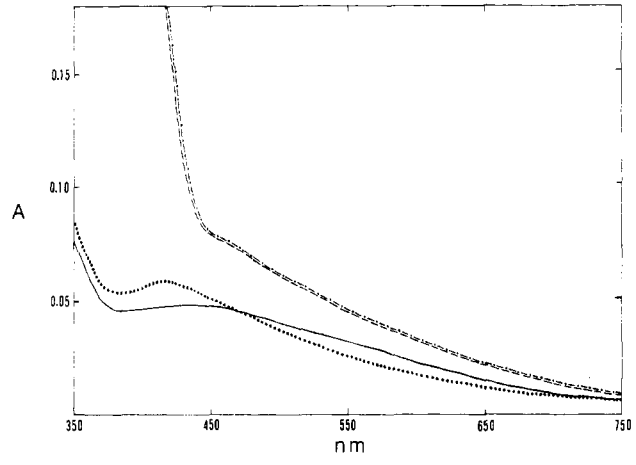


FIGURE 6: Equilibrium displacement of 3-fluoro-4-hydroxybenzoic acid with protocatechualdehyde. Curve 1 (—), spectrum of native PCD (0.8 mg/mL; 10 μM active sites); curve 2 (···), spectrum of native PCD at same concentration as in curve 1, with 27 μM 3-FHB present; curve 3 (- - -), spectrum of native PCD solution used to run curve 1 after addition of protocatechualdehyde to 2.5 mM; curve 4 (-·-), spectrum of solution used to run curve 2 after addition of 2.5 mM protocatechualdehyde. Curves are corrected for dilution.

stopped-flow studies confirmed that PCL completely displaces bound 3-FHB, the spectrum of the PCD·PCL complex being identical whether or not PCD had been initially complexed with 3-FHB (Figure 6).

As shown in Figure 1B, the k_{off} values for 3-FHB and 3-ClHB are very similar, both rising rapidly above pH 8.5. We have previously reported that the K_I values for 3-FHB are identical with K_D values obtained from spectral titrations, thus establishing that simple complex formation ($K_I = k_{off}/k_{on}$) is being reflected in the kinetically determined K_I values (May et al., 1978). Therefore, it is clear from a comparison of the K_I and k_{off} profiles in Figure 1 that the pH dependence for k_{on} is expected to be strikingly similar to that for k_{cat}/K_m in zwitterionic buffers, which we have already indicated probably reflects simply the pH dependence of k_1, the substrate association step. Thus, we again see that the ionization effects on substrate and inhibitor binding are very similar and do not reflect the relative acidity of the *p*-hydroxyl moiety. We note here that k_{on} for substrate and halohydroxybenzoates is far too fast for the association step to be followed directly in the

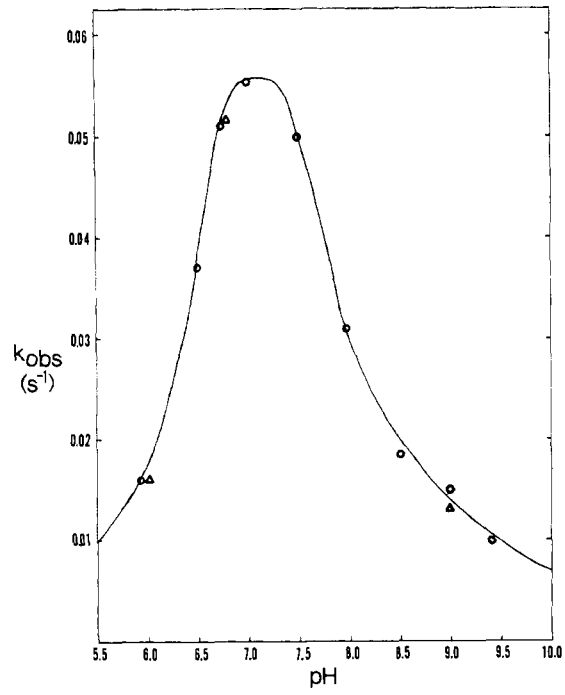


FIGURE 7: pH dependence of 4-nitrocatechol dissociation. Dissociation rate constants were measured as previously described (May et al., 1978) and as described under Materials and Methods. Solutions of PCD (~1 mg/mL; ~10 μM sites) were prepared in the appropriate buffers (0.05 M phosphate or Tris), precomplexed with 4-NC, and then mixed with solutions containing a large excess (>100-fold) of either 3-FHB (○) or 3-ClHB (△) in the same buffer in the stopped-flow spectrophotometer. First-order kinetics were observed, with the reaction being followed in the dual-wavelength mode at 550 and 430 nm. The curve shown in this plot is hand drawn for illustration.

stopped-flow spectrophotometer.

Kinetic Studies with 4-Nitrocatechol. We have previously reported (May et al., 1978) on preliminary results of kinetic experiments in which the chromophoric PCD inhibitor 4-nitrocatechol (4-NC) (Tyson, 1975) was displaced by 3-FHB. We remarked on the extremely slow dissociation rate for 4-NC and suggested that this effect was responsible for the complex behavior of this ligand which we observed in steady-state kinetic experiments. We have now further examined the displacement of 4-NC throughout the pH region from 6.0 to 9.5, shown in Figure 7. The dissociation rates observed in these experiments were identical at a given pH when either 3-FHB (○), 3-ClHB (△) or PCL (not shown) was used as the displacer, which provides further strong support that the conditions of eq 4 discussed above for displacement reactions are met. The profile for this reaction is bell-shaped, in sharp contrast to the k_{off} profile for the halohydroxybenzoate inhibitors.

In further contrast to the situation with the halohydroxybenzoates, association of 4-NC with PCD can be followed directly in the stopped-flow spectrophotometer. As shown in Figure 8, the observed kinetics of association depend on the wavelength used. When association was followed at 550 nm with a 700-nm reference wavelength, a simple first-order reaction was observed at pH 7.5, with an apparent rate constant of ~2 s^{-1}, which is independent of [4-NC]. On the other hand, when the sample monochromator was set on 430 nm (the absorption peak of the free 4-NC anion), a distinctly biphasic reaction was observed, in which the rate of the rapid phase was dependent on [4-NC] while the slow phase proceeded at the same rate as that observed at 550 nm, again being independent of [4-NC]. From a series of kinetic experiments, the second-order rate constant for the rapid phase was estimated

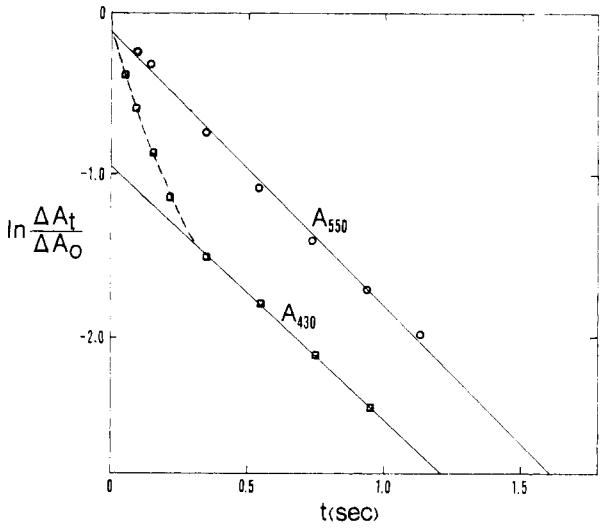


FIGURE 8: Association of 4-nitrocatechol with PCD. These experiments were carried out as described under Materials and Methods. PCD solutions (~1 mg/mL; ~10 μM sites) in 0.05 M Tris-HCl, pH 7.5, were mixed with solutions of 4-NC (100–500 μM) prepared in the same buffer. The reaction was followed in the stopped-flow spectrophotometer in the dual-wavelength mode at either 430 (□) or 550 (○) nm with a reference of 700 nm.

to be $\sim 1 \times 10^5$ M^{-1} s^{-1}. Thus, it is clear that the interaction of 4-NC with PCD is a complex process, and the kinetics are consistent with a rapid bimolecular association followed by a surprisingly slow formation of a second complex. A slow process of this type is seen neither in substrate (Fujisawa et al., 1971, 1972b) nor in halohydroxybenzoate association.

Discussion

We have previously demonstrated that 3-halo-4-hydroxybenzoates such as 3-FHB and 3-ClHB are potent inhibitors and active-site titrants which exhibit simple kinetic and spectral properties, and our comparative studies with 3- and 4-substituted hydroxybenzoates established the critical role of the 4-hydroxyl substituent in binding (May et al., 1978). Insight into the molecular details of metal participation in these binding interactions has been provided by our recent resonance Raman studies on resting PCD and both the E·S and E·I complexes (Felton et al., 1978). The iron atom in PCD is tyrosine ligated, an observation confirmed in two other laboratories (Keyes et al., 1978; Tatsuno et al., 1978) and consistent with CD studies (Hou, 1975), and we have been able to estimate that two tyrosines are involved in iron coordination. In the E·I complexes with 3-FHB and 3-ClHB, inhibitor interacts with the metal atom via Fe–O ligation without displacing either tyrosine, thus clarifying the critical need for a *p*-hydroxyl substituent for potent binding. In the case of substrate, the stable E·S species observed anaerobically by Raman exhibits iron chelation by the *o*-dihydroxy grouping, again with neither tyrosine ligand being displaced. In all probability, the carboxylate functionality of either substrate and inhibitor ionically interacts with a cationic group, either an arginine [by analogy with *p*-hydroxybenzoate hydroxylase (Shoun et al., 1979)] or possibly a lysine.

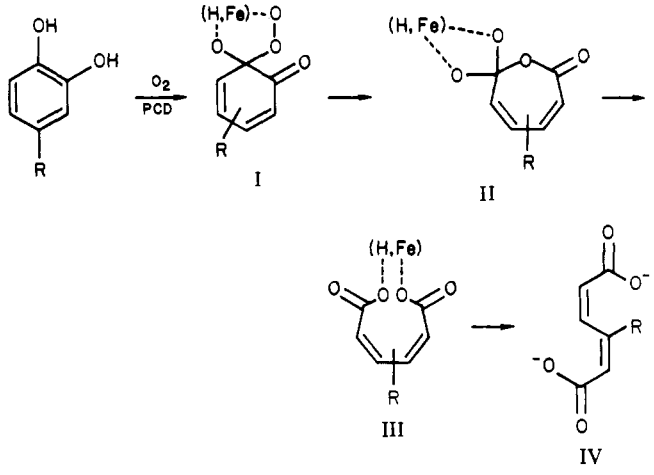
At first glance, it seems natural to conclude that since the E·I complexes of all inhibitors containing 4-OH substituents exhibit virtually identical spectral features (May et al., 1978), the increased inhibitor potency caused by 3-halo substitution is related to the greatly increased acidity of the *p*-hydroxyl. The data in this paper establish that prior ionization is not, in fact, responsible for the increased binding potency, as reflected in the K_I values for the halohydroxybenzoates, and furthermore that the ionization effects on substrate and inhibitor association rate constants are very similar. It seems reasonable to assume that the monodentate Fe–O ligation seen in E·I also occurs for substrate on the pathway toward chelation, which is the stable state actually seen in the Raman studies on the E·S complex (Felton et al., 1978). Thus, we conclude that the active site of PCD is set up to provide for facile removal of the proton from even a nonacidic *p*-hydroxyl such as those found in all known PCD substrates. This could be accomplished by the presence of a base (e.g., a histidine), which picks up the proton as the hydroxyl nears the coordination sphere of the iron, effectively lowering its pK_a. This picture then explains why PCD readily accommodates either the protonated or the anionic forms of the halohydroxybenzoates, the only difference in the respective complexes being the ionization state of this basic group. In order to accommodate the kinetic and spectral data, we conclude that the ionization state of this base has a negligible effect on either the spectral or the kinetic properties of the E·I complex. A strikingly similar conclusion has recently been reached regarding *p*-hydroxybenzoate hydroxylase, where a change in the ionization state of an interaction involving the *p*-hydroxy moiety of the substrate has a negligible effect on K_D (Shoun et al., 1979).

In view of the above conclusions, alternative explanations must be found for the high potency of the 3-halo-4-hydroxybenzoates. Our data clearly reveal a high sensitivity to the steric requirements of the substituents at position 3, but not at position 2, and thus interactions involving the substituent at position 3 likely play an important role. It is attractive to postulate that the 3-halogen substituents mimic the 3-OH moiety of substrates, particularly in the case of the isoelectronic fluorine, and thus are capable of increased binding interactions relative to the unsubstituted 4-hydroxybenzoic acid. The potential binding interactions which could contribute include hydrogen bonding, operative in the case of fluorine substituents, or possibly "pseudochelation" of the iron (Wong et al., 1976).

The steady-state kinetic data with the substrate, PCA, give k_{cat} values which exhibit a simple sigmoidal pH dependence, with a pK_a of ~7. Since at pH 7.5 $k_{cat} = k_5$ (Fujisawa et al., 1971), these data reflect an ionization effect on the breakdown of E·S·O_2, so long as $[O_2] \gg K_m^{O_2}$ and the steady-state is maintained over the entire pH range. That this is indeed the case is strongly supported by our experiments with DHPP, where direct observations of k_5 affords an analogous pH dependence. Thus, an ionization near pH 7 facilitates the final step in PCD catalysis, and it is instructive to consider this result in light of current thinking about the mechanism of action of this enzyme.

Hamilton (1974) has proposed a mechanism for aromatic dioxygenases involving ketonization of a phenolic hydroxyl to supply electron density for attack of oxygen at the adjacent carbon to give an α-keto hydroperoxide (I). Subsequently, this species could rearrange to a pseudoanhydride (II) and then collapse directly to the ring-opened product (III).

Although Que et al. (1977) favored structure I for the spectrally distinct E·S·O_2 species based on analogy with oxyhemerythrin, recent work by Nakata et al. (1978) demonstrates that the E·S·O_2 complex is almost certainly closer to the product than structure I, since β-carboxyethylmuconic acid is quantitatively recovered from E·S·O_2 under both acidic and neutral conditions. We note that it is highly unlikely that structure I would give quantitative conversion to only the muconic acid under these conditions, since recent work by Nishinaga et al. (1978) has shown that a variety of products



can be formed from analogous α-keto hydroperoxides under acid and basic conditions. Therefore, we conclude that although structure I may well be along the reaction pathway, catalysis has proceeded at least to the Hamilton intermediate (II) at the stage of the spectrally distinct E·S·O_2 complex, since II would be expected to decompose readily to the muconic acid under the conditions of Nakata's experiments. Equally consistent with these data is the postulate that E·S·O_2 is actually structure III, a true E·P complex where complete ring opening has already occurred and product release is the rate-determining step. The argument of Que et al. (1976) against the E·P complex (III) based on adding product and product analogues to PCD and examining spectral properties is not sufficient to rule out this possibility. Elvidge et al. (1950) have pointed out that muconic acids exist in solution in the s-trans (IV) configuration, and since the incipient enzymatic product is undoubtedly confined to the s-cis configuration, it is highly unlikely that simply adding product to free PCD would give back the E·P complex formed during catalysis. Strong support for this conclusion is provided by the fact that product inhibition of PCD catalysis is so weak as to be virtually nonexistent (Hori et al., 1973), and we observe no deviation from first-order decomposition of E·S·O_2 (from DHPP) even after 250 μM product has accumulated.

Our finding that ionization facilitates breakdown of the E·S·O_2 complex is also consistent with this species being either the Hamilton pseudoanhydride (II) or a simple E·P complex (III). In II, one of the *gem*-diol oxygens is presumably Fe ligated and the other is capable of being protonated. There is direct analogy for ionization facilitating breakdown of such a species from the studies of Seltzer & Stevens (1968) with β-acetylacrylate pseudoacid. Alternatively, if decomposition of II at the active site is catalyzed by a general base, the pK_a of this species could be reflected in our pH profile. Our data are also obviously consistent with structure III in that ionization of some cationic species at the active site, with a pK_a near 7 (histidine?) would facilitate release of the anionic product into solution.

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