Hydroxybenzoyl-CoA Reductase: Coupling Kinetics and Electrochemistry To Derive Enzyme Mechanisms†

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ABSTRACT: Hydroxybenzoyl-CoA reductase (HBCR) is an iron−sulfur protein that is involved in the metabolism of aromatic compounds. It catalyzes the two-electron reduction of hydroxybenzoyl-CoA to benzoyl-CoA. In the work described here, kinetic schemes were derived for HBCR and for several classes of redox enzymes and redox-activated enzymes. Introduction of the Nernst equation into the rate laws led to the development of novel relationships between the ambient redox potential, the midpoint potential of the enzyme active site, and the kinetic parameter, *V/K*. By coupling electrochemistry and steady-state kinetics, mechanistic information could be obtained that could not be determined by either method alone. For HBCR, the relationship between the kinetic parameter *V/K* and the ambient electrochemical potential of the assay mixture was found to be: apparent $V/K_m = V_{max}/\left\{K_m\left\{1 + \exp\left[\frac{nF}{RT}(E - E^\circ_e)\right]\right\}\right\}$, where n is the number of electrons involved in the redox process, F is the Faraday constant, R is the gas constant, T is the temperature in K, E is the applied potential, and E°_e is the redox potential of a redox-active catalytic site on the enzyme. Coupling kinetics with electrochemistry yielded the E°_e (−350 mV vs NHE) for HBCR and maximum values under optimal redox conditions for k_{cat} and k_{cat}/K_m (9 s^{-1} and 1.8 × 10^5 M^{-1} s^{-1}, respectively). In addition, theory was developed that could distinguish a single two-electron transfer mechanism from one involving two successive one-electron transfers. HBCR was found to be in the latter class. Interestingly, the derived mechanism for HBCR is similar to that of the Birch reduction, the classical organic chemical reaction for reductive dehydroxylation of phenolic compounds. The methodology described here represents a novel approach that should help elucidate the mechanisms of other oxidoreductase and redox-activated enzymes.

Hydroxybenzoyl-CoA reductase (HBCR)[1] is involved in the anaerobic oxidation of aromatic compounds to CO_2. This is an important part of the global carbon cycle because lignin represents a rich energy source for organisms. The anaerobic breakdown of monomeric aromatics derived from lignin can occur at the expense of a variety of electron acceptors (Evans & Fuchs, 1988). Most naturally occurring aromatics contain hydroxyl and carboxylate functionalities. During anaerobic biodegradation, the carboxyl groups are thioesterified with CoA and the hydroxyl groups are removed by reductive cleavage. HBCR catalyzes the two-electron reductive dehydroxylation of *p*-hydroxybenzoyl-CoA to form benzoyl-CoA. The further metabolism of benzoyl-CoA to acetyl-CoA involves aromatic ring reduction followed by a series of steps that resemble fatty acid degradation (Evans & Fuchs, 1988). HBCR has been purified from a denitrifying *Pseudomonas* sp. (Brackmann & Fuchs, 1993). It was shown to have an $\alpha_2\beta_2\gamma_2$ stoichiometry with 75, 35, and 17 kDa subunits. There are 12 Fe and 12 inorganic sulfide (per mol of 260 kDa hexamer). The Fe and S^{2-} content suggests that the iron may be in the form of Fe-S clusters, and there is no spectral evidence for heme iron.

The enzymatic mechanism of HBCR is interesting because reductive dehydroxylation of an aromatic ring is a difficult chemical reaction. One mechanism for such a reaction in the organic chemical literature is known as the Birch reduction. This reaction appears to occur through two discrete one-electron steps in which the first reduction generates a radical anion. Mechanistic information on the biochemical process is lacking.

In this manuscript, we have probed the mechanism of HBCR using an approach that combines steady-state enzyme kinetics with electrochemistry. Standard procedures were used to develop possible kinetic schemes and the corresponding rate laws. The Nernst equation was then introduced into the rate law to derive a general relationship between the applied potential of the assay mixture and the kinetic parameter *V/K*. This novel methodology was used to optimize the redox conditions for assaying the enzyme, define the redox potential (E°) of the active site center, and evaluate the order of electron transfer steps relative to the substrate-binding step. This work represents a significant advance in the ability of controlled potential enzymology (Lu *et al.*, 1990; Lu & Ragsdale, 1991) to furnish mechanistic information and complements the arsenal of spectroscopic, kinetic, and electrochemical methods available to characterize

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[1] Abbreviations: HBCR, hydroxybenzoyl-CoA reductase; E°_e, the redox potential for the active site; $E_{1/2}$, half-turnover potential.

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enzymes. The method is general for enzymes that catalyze redox reactions or undergo oxidative or reductive activation.

MATERIALS AND METHODS

Cell Culture and Enzyme Preparation. Pseudomonas sp. was grown at 28 °C under anaerobic conditions, with 5 mM 4-hydroxybenzoate and 20 mM nitrate (Brackmann & Fuchs, 1993). HBCR was purified as described (Brackmann & Fuchs, 1993).

Synthesis of 4-Hydroxybenzoyl-CoA. Preparation of 4-hydroxybenzoyl-CoA followed a procedure for synthesis of thioesters developed by M. Sappelt (University of Konstanz, Germany). Reaction of 4-hydroxybenzoic acid with CoA (Li salt) was performed in dry tetrahydrofuran under a nitrogen atmosphere in the presence of ethyl chloroformate and triethylamine through the formation of a mixed anhydride. Hydroxybenzoyl-CoA was purified by HPLC with an Ultrasphere Bondclone 10-C_{18} reverse-phase column (Phenomenex) (Merkel *et al.*, 1989). A linear gradient (1 mL min^{-1}) of acetonitrile (solvent A) and 0.2 M ammonium acetate (solvent B) was run from 0.5% to 25% solvent A in 25 min followed by a linear gradient of 25−75% solvent A in 5 min. Hydroxybenzoyl-CoA eluted at 35 min and was lyophilized.

Spectrophotometric Assay. Reduction of 4-hydroxybenzoyl-CoA was coupled to the oxidation of reduced benzyl viologen. The 1 mL standard assay mixture contained 0.1 M potassium phosphate, pH 7.0, 5 mM $MgCl_2$, 0.5 mM benzyl viologen, 0.01−0.2 mM 4-hydroxybenzoyl-CoA, 0.01−0.02 mg of protein, and enough sodium dithionite to reduce the benzyl viologen to give an absorption of ~2.0 at 578 nm. The reaction was run at 30 °C, started by adding 4-hydroxybenzoyl-CoA, and monitored spectrophotometrically at 578 nm where the viologen radical has maximum absorbance (ϵ_{578} = 10 000 M^{-1} cm^{-1}). Two moles benzyl viologen are oxidized per 1 mol of 4-hydroxybenzoyl-CoA reduced (Brackmann & Fuchs, 1993).

Spectroelectochemical Experiments. An anaerobic spectroelectrochemical cell with a typical three-electrode circuit was constructed that included a gold foil working electrode (area = 2 cm^2), silver/silver chloride reference electrode, and counter electrode. The main body of the cell was quartz and had a minimum sample requirement of 1.8 mL. A small stirrer was constructed from a small magnet sealed in glass or poly(ethylene). The optical path length of the cell was 1 cm. All experiments were performed at 30 °C. The auxiliary and reference electrodes were stored anaerobically for 12 h prior to the experiment. The system was closed and made anaerobic by cycling with vaccum and deoxygenated purified nitrogen for at least 1 h. Oxygen was removed by passing the nitrogen over a heated copper catalyst. The reaction mixture contained 0.1 M potassium phosphate, pH 7.0, 5 mM $MgCl_2$, and 0.1−1 mM of one of the following anaerobically prepared redox dyes: anthraquinone 1,5-disulfonate (E'°_7 = −0.174 V vs NHE), phenosafranine (E'°_7 = −0.252) (Clark, 1960), benzyl viologen (E'°_7 = −0.358), or methyl viologen (E'°_7 = −0.449) (Steckhan & Kuwana, 1974). After introduction of the dye, the system was cycled several additional times with vacuum/nitrogen. A potentiostat (BAS CV-27) was used to initially poise the potential at a value more negative than the desired equilibrated potential and then turned off. When the system reached equilibrium, the enzyme was added to the cell with a gastight Hamilton syringe under a positive gas flow, and the system once again was allowed to reach equilibrium. Equilibrium was defined by the absence of change in the UV−visible spectrum and the ambient potential. The ambient potential was monitored by a voltmeter (Fluke 85 multimeter) connected across the working and reference electrodes. The reaction was started by adding 4-hydroxybenzoyl-CoA. Spectra were acquired using a DU 700 diode array spectrophotometer (Beckman).

An increase in the redox potential during the reaction is expected since the potentiostat was turned off after the system was poised at an initial given potential and 4-hydroxybenzoyl-CoA underwent reduction. As 4-hydroxybenzoyl-CoA is reduced, the reduced dye is consumed. This inevitably leads to an increase in the ratio of oxidized/reduced dye and an increase in the potential. When the initial applied potentials were −0.328, −0.345, and −0.357, the potentials increased by 9, 11, and 19 mV, respectively, during the reactions (see Figure 1a).

Analytical Methods. The kinetic parameter V_{max}/K_m was obtained by fitting the decreasing absorbance of reduced dye versus time to eq 1, which is the exponential form of the Michaelis−Menten equation for a first-order or pseudo-first-order reaction. The value for $V_{max}$was obtained by following

$$A = (A_0 - A_i)\exp(-V_{max}t/K_m) + A_i \quad (1)$$

$$A = (A_0 - A_i)\exp\{(V_{max}/K_m)[(A_0 - A)/V_{max} - t]\} + A_i \quad (2)$$

the experiment to completion and fitting the data to the integrated form of the Michaelis−Menten equation (eq 2). This equation can be used when the decrease in velocity with time results only from decreased saturation of the enzyme, not from product inhibition or approach to equilibrium (Segel, 1975). In all of our experiments, hydroxybenzoyl-CoA was completely converted to benzoyl-CoA, demonstrating the irreversibility of the reaction. In addition, the kinetic trace when substrate is added again to a completed reaction was identical with the trace for the first reaction. These results indicate that the HBCR reaction satisfies the conditions for treating the data by either eq 1 or 2.

RESULTS

Controlled Potential Kinetics of HBCR. The effect of changing the applied electrochemical potential on the rate of the HBCR reaction was studied. A solution containing enzyme and mediator was poised at different potentials in a spectroelectrochemical cell, substrate was added to initiate the reaction, and dye mediator oxidation was followed (Figure 1). A large window of potentials was made accessible by the use of redox dyes ranging from anthraquinone 1,5-disulfonate to methyl viologen. We also followed reduction of hydroxybenzoyl-CoA that has an absorption peak at 299 nm (Webster *et al.*, 1974). At this wavelength, benzyl viologen has only a weak absorbance. From the absorption changes at 578 and 299 nm during the reaction, it was found that 2.0 ± 0.2 mol of benzyl viologen were oxidized per 1 mol of 4-hydroxybenzoyl-CoA reduced. The concomitant formation of benzoyl-CoA has been previ-

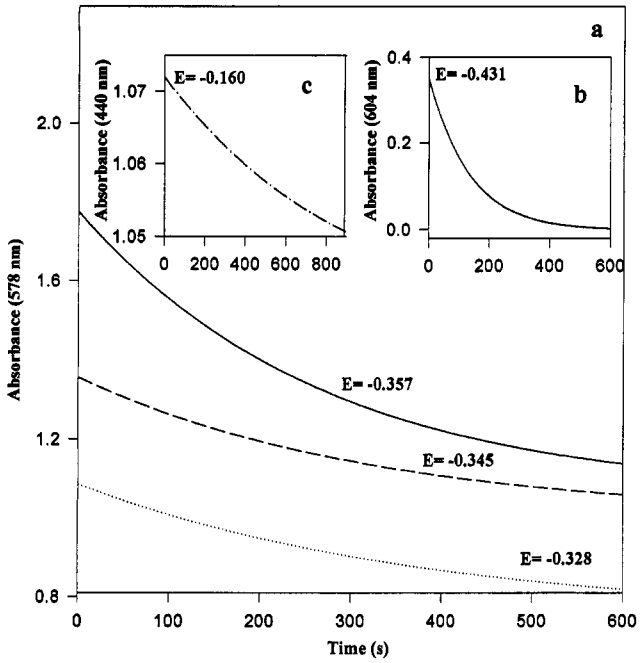


FIGURE 1: Spectroelectrochemical assay for HBCR reaction at different poised reduction potentials. The reaction mixture contained potassium phosphate buffer (0.1 M, pH 7.0), $MgCl_2$ (5 mM), HBCR (85 nM in $\alpha\beta\gamma$), and 4-hydroxybenzoyl-CoA (0.02−0.04 mM). The redox dyes and applied potentials for the reactions were (a) benzyl viologen (0.4 mM) at −0.328, −0.345, and −0.357 V vs NHE, (b) methyl viologen (0.13 mM) at −0.431 V vs NHE, and (c) anthraquinone 1,5-disulfonate (0.4 mM) at −0.160 V vs NHE. The reactions were carried out at 30 °C in a spectroelectrochemical cell as described in Materials and Methods. With anthraquinone, the rates were low enough that 2-fold higher enzyme concentration was used.

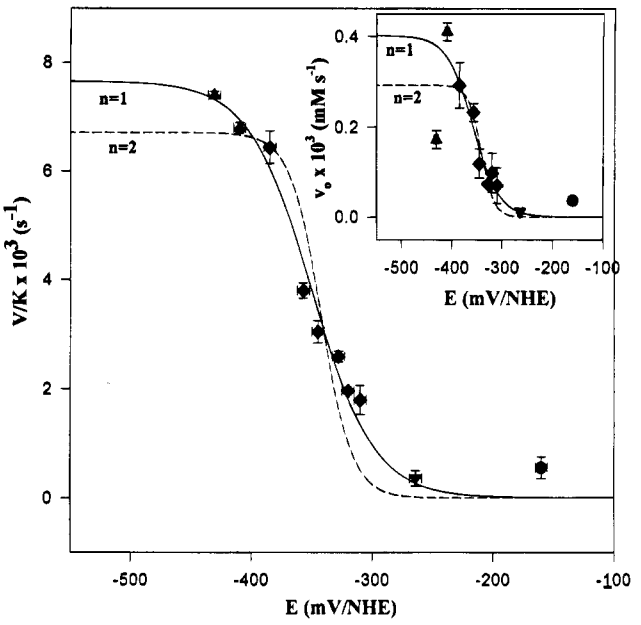


FIGURE 2: Dependence of the kinetic parameter V_{max}/K_m and initial velocity on the electrochemically applied potential. Shown is the fit of the experimental data to eq 4a (—), eq 4b with $n = 2$ (— — —), and eq 5 (indistinguishable from the fit to eq 4b). The error bars correspond to the standard deviation calculated from the fitting procedure. Symbols refer to experiments using methyl viologen (▲), benzyl viologen (◆), phenosafranine (▼), and anthraquinone 1,5-disulfonate (●) as mediators.

ously demonstrated by isolation of the CoA thioesters by HPLC (Glöckler *et al.*, 1989).

It was established that use of the integrated Michaelis−Menton equation to study the mechanism of HBCR was appropriate (see the methods section). Below 200 μM hydroxybenzoyl-CoA, the value of V_{max}/K_m was independent of the concentration of hydroxybenzoyl-CoA and, therefore, was an apparent first-order rate constant. For example, at 20 and 200 μM, the values for V_{max}/K_m were 1.80 (±0.27) × 10^{-3} and 2.01 (±0.10) × 10^{-3} s^{-1}, respectively, at an applied potential of −0.310 V versus NHE.

The values of V_{max}/K_m for hydroxybenzoyl-CoA (Figure 2) and the initial velocities (Figure 2, inset) that were obtained from the kinetic traces shown in Figure 1 were plotted versus applied potential. The values of both V_{max}/K_m and v_o increased as the potential was stepped to more negative values. In order to derive a theoretical relationship between V_{max}/K_m and the applied potential, it was necessary to define the steady-state mechanism of the reaction. Several lines of evidence indicated that HBCR uses a ping-pong mechanism. First, the V_{max}/K_m was found to be a function of the applied potential and independent of the nature of the redox dye. The mediators that were used included methyl and benzyl viologen, which transfer one electron, and anthraquinone disulfonate and phenosafranine, which are two-electron donors. Second, when the reaction was run at a constant applied potential, the V_{max}/K_m for hydroxybenzoyl-CoA determined with benzyl viologen as mediator was the same as that obtained with methyl viologen. These results are consistent with a ping-pong mechanism in which the chemical nature of the electron donor used for reduction of the enzyme in the first half-reaction would not affect the rate of reduction of the substrate in the second half-reaction.

Effect of Product on the V/K for 4-Hydroxybenzoyl-CoA

Inhibition by Benzoyl-CoA. To study product inhibition by benzoyl-CoA, the applied potential and the concentrations of oxidized and reduced benzyl viologen (0.25 mM each) and hydroxybenzoyl-CoA (0.025 mM) were constant. As the concentration of benzoyl-CoA was increased from 0 to 1 mM, both the V_{max}/K_m for hydroxybenzoyl-CoA (Figure 3) and V_{max}(Figure 4) decreased. K_i values derived from plots of V_{max}/K_m and V_{max}were 0.12 and 0.37 mM, respectively. Therefore, benzoyl-CoA can be classed as a mixed-type inhibitor (Table 1). In the absence of benzoyl-CoA, the K_m for hydroxybenzoyl-CoA was 0.06 (±5%) mM, and at 0.2 and 0.6 mM benzoyl-CoA, the K_m values were 0.09 (±5%) and 0.21 (±12%) mM, respectively.

Inhibition by Oxidized Dye Mediator. That benzoyl-CoA was a mixed inhibitor with respect to hydroxybenzoyl-CoA was consistent with a ping-pong mechanism; however, in order to fully define the mechanism, it was necessary to perform product inhibition studies with the other product, oxidized dye mediator. One cannot study the singular effect of increasing the concentration of oxidized or reduced dye on the reaction because altering the ratio of oxidized/reduced species changes the applied potential. This was problematic because the reaction rate was highly sensitive to changes in the potential. We attempted to determine the effect of oxidized redox mediator on the rate by maintaining the concentration of hydroxybenzoyl-CoA at 10 μM and the applied potential at −0.358 V where the concentrations of reduced and oxidized benzyl viologen are equivalent. As the total concentration of benzyl viologen was increased from

Table 1: Product Inhibition Patterns[a]

inhibitor	substrate varied	substrate fixed (unsat)	predicted pattern ternary complex	predicted pattern ping-pong	observed pattern
Bz-CoA	HBz-CoA	^{red}BV	M	M	M $K_i = 0.12 \pm 0.03$ mM $K_i' = 0.37 \pm 0.09$ mM
^{ox}BV	HBz-CoA	^{red}BV	M	C	C 0.11 ± 0.02 mM

[a] M and C refer to mixed and competitive modes of inhibition, respectively.

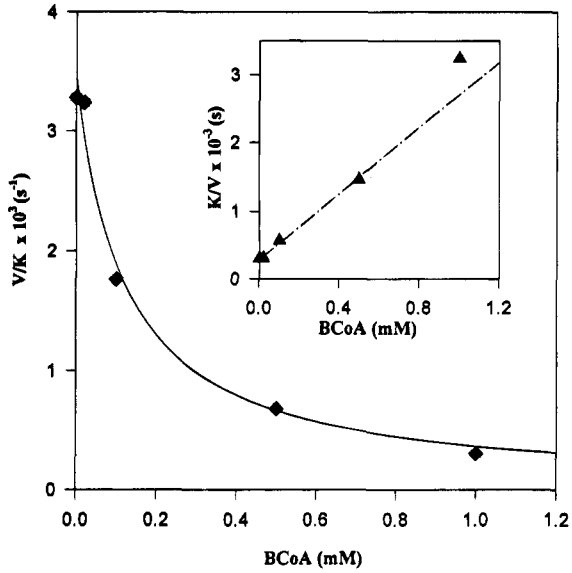


FIGURE 3: Product inhibition by benzoyl-CoA. The V_{max}/K_m for 4-hydroxybenzoyl-CoA was determined at varied concentrations of benzoyl-CoA. The reaction mixture contained potassium phosphate (0.1 M, pH 7.0), $MgCl_2$ (5 mM), benzyl viologen (0.5 mM), HBCR (85 nM, $\alpha\beta\gamma$), 4-hydroxybenzoyl-CoA (0.025 mM), and benzoyl-CoA (0−1 mM). These experiments were run at a constant applied potential of −0.358 V vs NHE and a temperature of 30 °C.

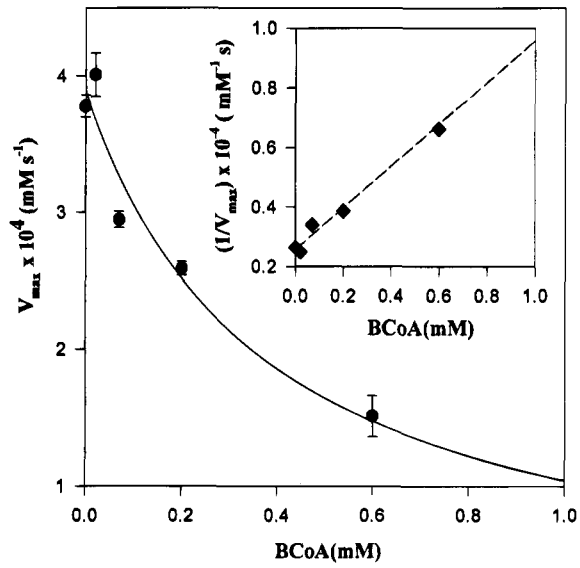


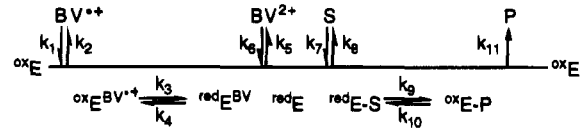
FIGURE 4: Product inhibition by benzoyl-CoA. The V_{max} was determined at varying concentrations of benzoyl-CoA. These experiments were performed under the same conditions as described in the legend to Figure 3. V_{max} is expressed in mM relative to benzyl viologen.

0.1 to 2 mM (the ratio of oxidized/reduced was constant), the V_{max}/K_m for hydroxybenzoyl-CoA decreased significantly, yielding a K_i of 0.11 mM (Figure 5). The value of the apparent V_{max} increased slightly with increasing concentration of viologen (Figure 6) because viologen was not at completely saturating concentrations. However, it was most important that V_{max} did not decrease. This indicates that the K_m for the reduced dye is much smaller than the K_i' (inhibition constant for the E−S complex) for the oxidized dye. Our results indicate that oxidized viologen binds only to the free enzyme, resulting in a decrease in the V_{max}/K_m. Thus, the oxidized mediator product can be classed as a competitive inhibitor with respect to hydroxybenzoyl-CoA. To summarize the product inhibition results, competitive inhibition by oxidized benzyl viologen and mixed inhibition by benzoyl-CoA with respect to hydroxybenzoyl-CoA are consistent with a ping-pong mechanism.

Measurement of V/K at Varied Applied Potentials. The results of steady-state kinetic studies indicated that HBCR follows a ping-pong mechanism. However, by coupling kinetics with electrochemistry, we found that certain mechanisms that appear to be ping-pong actually are not.

Since the reduction of 4-hydroxybenzoyl-CoA to benzoyl-CoA involves two electrons, two types of mechanisms were considered. On the one hand, substrate could bind to the enzyme at the one-electron-reduced state (Scheme 1). In

Scheme 1



this case, when catalysis takes place, a radical species would be transiently generated, *i.e.*, after one-electron transfer to the substrate from ^{red}E, P would be a radical that could dissociate (as shown in the scheme) or undergo further reaction (as considered below). For HBCR and most enzymatic reactions, the radical species would have to undergo another one-electron reduction. Although this scheme could be considered heuristic, we demonstrate below that the mechanism of HBCR effectively reduces to Scheme 1 (see Discussion). The cell reaction for the first step in such a mechanism corresponds to the following equilibrium:

$$^{ox}E + BV^{\bullet+} \rightleftharpoons {}^{red}E + BV^{2+}$$

Alternatively, two electrons could be transferred to the enzyme before substrate binds (Scheme 2). In this type of mechanism, the cell reaction corresponds to the equilibrium:

$$^{ox}_{ox}E + 2BV^{\bullet+} \rightleftharpoons {}^{red}_{red}E + 2BV^{2+}$$

Scheme 2 describes a classical ping-pong mechanism. We derived relationships between V_{max}/K_m and the applied potential for these mechanisms (see Appendix). In these

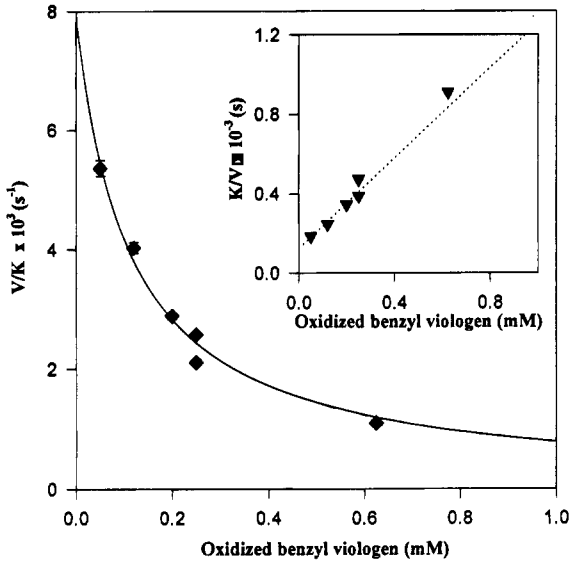


FIGURE 5: Product inhibition by oxidized benzyl viologen. The V_{max}/K_m for 4-hydroxybenzoyl-CoA was determined at varying concentrations of benzyl viologen. The assay mixture contained potassium phosphate (0.1 M, pH 7.0), $MgCl_2$ (5 mM), HBCR (85 nM, $\alpha\beta\gamma$), 4-hydroxybenzoyl-CoA (0.010 mM), and benzyl viologen (0.1−2 mM). The applied potential was −0.358 V vs NHE, and the assay temperature was 30 °C. The experiments with concentrations of benzyl viologen of 1.25 and 2 mM were carried out in a cell with an optical path length of 0.2 cm.

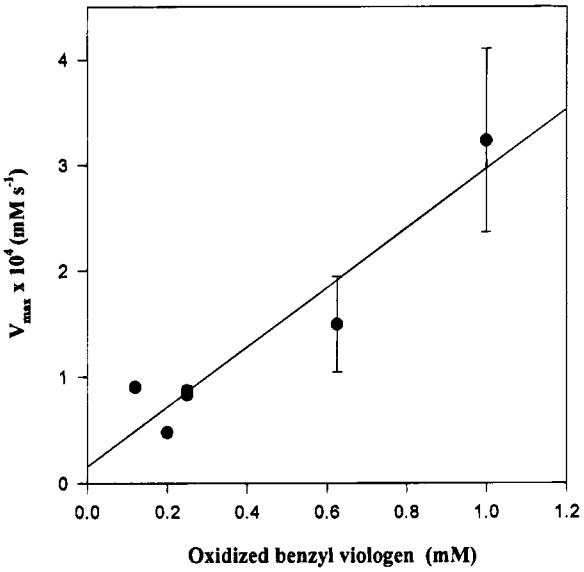
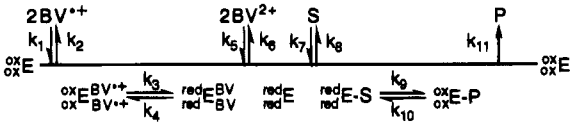


FIGURE 6: Product inhibition by oxidized benzyl viologen. The $V_{max}$was determined at varying concentrations of benzyl viologen. The experimental conditions were as described in the legend to Figure 5.

Scheme 2



equations, n is the number of electrons involved in the redox process, F is the Faraday constant, R is the gas constant, T is the temperature in K, E is the applied potential, and $E°_e$ is the formal reduction potential of the site undergoing redox chemistry. Since, in Scheme 1, 1 equiv of reduced dye mediator binds to the enzyme before substrate binds, the dependence of V_{max}/K_m on applied potential is given by eq 3a with $n = 1$.

$$\frac{V}{K} = \frac{\left(\frac{V}{K}\right)\text{max}}{1 + \frac{[O]}{K'^{O}_{i}} + e^{\frac{nE}{RT}(E-E°_e)}} \quad (3a)$$

"O" represents the oxidized dye mediator and $(V/K)_{max}$ designates the V/K under optimal redox conditions, in this case at infinite negative potentials. For the mechanism depicted in Scheme 2, the relationship between V_{max}/K_m and applied potential is given by eq 3b with $n = 2$.

$$\frac{V}{K} = \frac{\left(\frac{V}{K}\right)\text{max}}{1 + \frac{[O]^2}{K'^{O}_{i2}} + e^{\frac{nE}{RT}(E-E°_e)}} \quad (3b)$$

In order to discriminate between these two mechanisms, the results of the kinetic studies at different potentials were fit separately to eqs 3a and 3b. Knowing the concentration of oxidized dye for each experiment and the applied potential, we were able to determine $(V/K)_{max}$, $E°_e$, K'^{O}_{i} and K'^{O}_{i2}. We found that the terms $[O]/K'^{O}_{i}$ and $[O]^2/K'^{O}_{i2}$ were insignificant and eqs 3a and 3b could be simplified to give eqs 4a and 4b.

$$\frac{V}{K} = \frac{\left(\frac{V}{K}\right)\text{max}}{1 + e^{\frac{E}{RT}(E-E°_e)}} \quad (4a)$$

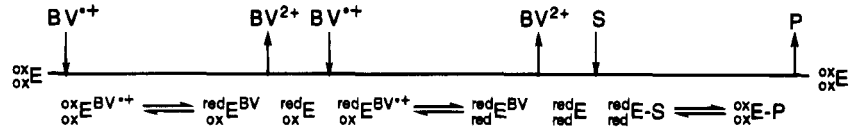
$$\frac{V}{K} = \frac{\left(\frac{V}{K}\right)\text{max}}{1 + e^{\frac{2E}{RT}(E-E°_e)}} \quad (4b)$$

Figure 2 shows the fits of the experimental data to eqs 4a (solid line) and 4b (hashed line). The best fit is obtained for $n = 1$ with values for $(V/K)_{max}$ of 7.67 (±0.33) × 10^{-3} s^{-1} and $E°_e$ of −0.350 ± 0.004 V vs NHE. The $(V/K)_{max}$ represents the V/K when $E \ll E°_e$, and the $E°_e$ is the redox potential of a site on the enzyme that catalyzes the reductive dehydroxylation of 4-hydroxybenzoyl-CoA. The K_m for hydroxybenzoyl-CoA was 50 μM, and maximum values for k_{cat} and k_{cat}/K_m were calculated to be 9 s^{-1} and 1.8 × 10^5 M^{-1} s^{-1}.

In the inset to Figure 2, the initial velocity is plotted versus potential. Because the initial velocity depends on both the nature and concentration of both substrates, only the data obtained using a single dye mediator were included in the fit. The values for $(v_o)_{max}$ and $E°_e$ were 0.402 (±0.072) × 10^{-3} mM s^{-1} and −0.357 ± 0.010 V vs NHE. As with the V/K data, the best fit is obtained for an $n = 1$ process.

Another mechanism can be considered in which the enzyme undergoes two successive one-electron transfers before substrate binds (Scheme 3). This also is a classical ping-pong reaction. In this case, one can consider two redox couples, $E°_1$ and $E°_2$, for the two redox reactions. $E°_1$ defines the first and $E°_2$ the second one-electron reduction of the

Scheme 3



enzyme. The dependence of V/K on applied potential can be derived for such a reaction and is expressed in eq 5.

$$(V/K_m)_{app} = \frac{(V/K_m)_{max}}{1 + 10^{[(2E-E°_1-E°_2)/60]} + 10^{[(E-E°_2)/60]}} \quad (5)$$

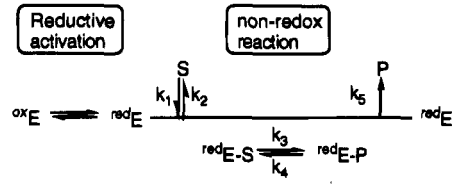
A best fit fit to eq 5 yields a value for $E°_2$ (−262 mV) more positive than $E°_1$ (−420 mV). Equation 5, then, practically reduces to eq 4b with $n = 2$. As shown on the fit to eq 5 in Figure 2, this does not match the data as well as the fit to eq 4.

DISCUSSION

Redox centers in proteins play important roles in catalysis, electron transfer, and regulation of gene expression and enzyme activity. Enzymes such as HBCR undergo redox changes at each turnover cycle. The activities of many other enzymes that do not catalyze redox reactions depend upon their redox state. The effect of altering the applied potential on the initial velocity of a reductively activated enzyme that performs a nonredox reaction has been studied (Lu *et al.*, 1990). The initial velocity followed a profile that could be fit to the Nernst equation to yield the half-turnover potential, $E_{1/2}$, which was defined as the applied potential where the velocity was half-maximal. Such a treatment could be connected to a two-state model of enzyme activation, as, for example, in the pH titration of an enzymic active site residue. The $E_{1/2}$ is correlated to the formal reduction potential, $E°_e$ (called the redox potential in this paper), for the active site, but quantitatively, the relationship between $E_{1/2}$ and $E°_e$ depends on the thermodynamics and kinetics of the coupled chemical reaction (Bard & Faulkner, 1980). For example, when a chemical reaction is coupled to a redox reaction (*i.e.*, one form of the redox couple selectively participates in the reaction), a shift in the $E_{1/2}$ of the redox couple is observed. If the reaction occurs selectively with the reduced component of a redox couple, the $E_{1/2}$ will be more positive than the $E°_e$ for the redox center. Even the binding of a ligand can alter the $E_{1/2}$.[2]

Kinetic schemes and their corresponding rate laws were derived for two major classes of redox reactions. The Nernst equation was then introduced into the rate laws to formulate relationships betwen the kinetic parameter V/K and the applied potential of the reaction mixtures. Fundamentally, this substitution is similar to the introduction of a pH titration expression into the rate law when an enzyme equilibrates between an active and inactive state as a function of pH.

Scheme 4



The relationships between pH and apparent V/K and V have been important in determining the nature of the pH dependence of enzyme reactions. Similarly, we consider that the relationship between the ambient electrochemical potential and these parameters could enhance our understanding of the nature of oxidoreductases and redox-activated proteins. The methodology and theory should be general for all oxidoreductase enzymes and will likely impact future mechanistic studies by enzymologists.

In the equations below, the V/K is called an apparent value when it is a function of the applied potential and the $(V/K)_{max}$ refers to the value at an optimum redox potential. Scheme 4 describes the simplest class of potential-dependent reactions. Here the enzyme undergoes redox activation and then catalyzes a nonredox chemical reaction. In this case, the coupled reaction occurs selectively with one component of the redox couple. One can derive eq 6 (see Appendix) for this scheme, where ^{ox}E and ^{red}E are the oxidized and reduced enzyme.[3]

$$v = \frac{V_{max}S}{K_m\left(1 + \frac{^{ox}E}{^{red}E}\right) + S} \quad (6)$$

After including the Nernst equation, eq 7 can be written.

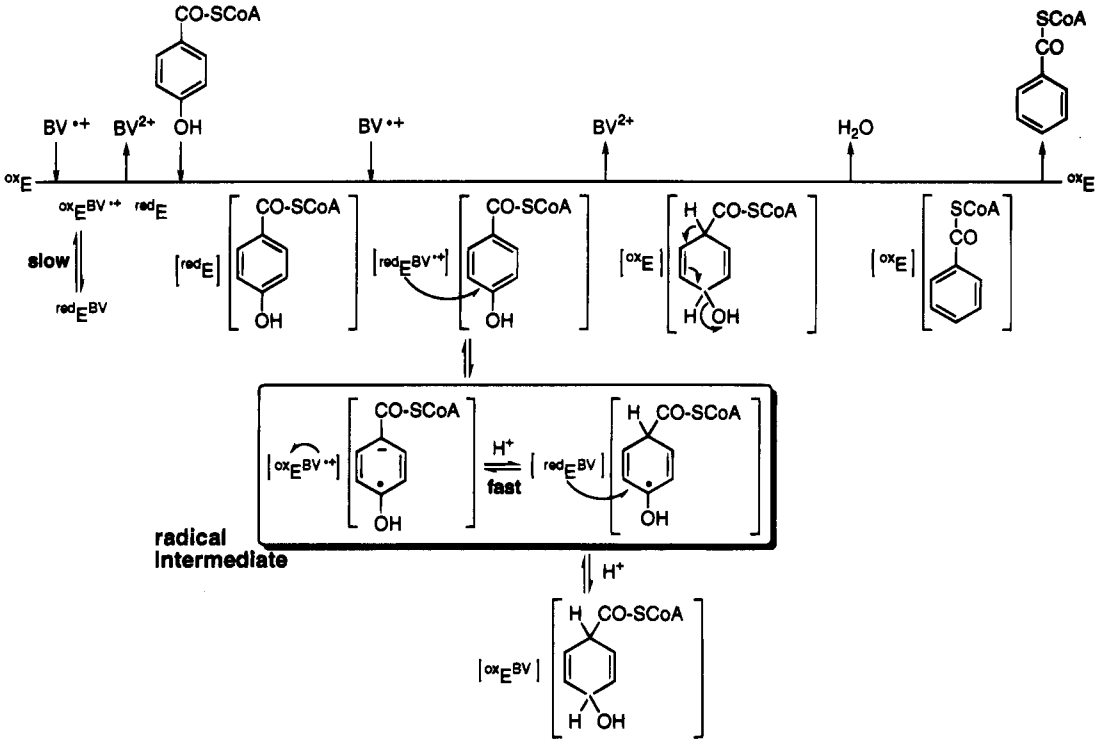
$$(V/K_m)_{app} = \frac{V_{max}}{K_m\left\{1 + \exp\left[\frac{nF}{RT}(E - E°_e)\right]\right\}} \quad (7)$$

We found that it was important to determine the effect of ambient potential on the kinetic parameter V/K instead of the initial velocity of the enzyme. Reasons for using basic kinetic constants instead of initial velocities are similar to those described for studying the effects of pH, inhibitors, activators, and ions on enzymes. In the case of redox reactions, there are additional advantages. In a ping-pong mechanism, the V_{max} and the initial velocity are dependent on the nature and concentration of the electron donor or acceptor. To obtain a full redox titration, it is important to vary the ambient potential over a ~120 mV range. Since redox buffers are useful only near their redox potentials, several mediators with different redox potentials must be used. Although, for HBCR, we found that the fits to both

[2] Examples include the general acyl-CoA dehydrogenase where binding of acyl-CoA substrates increases the midpoint potential of the flavin by over 100 mV (Lenn *et al.*, 1990) and nitrogenase where the oxidized form of the iron protein of nitrogenase binds Mg-ATP 100-fold tighter than the reduced enzyme (Watt *et al.*, 1986). If the reduced form of a redox center has higher affinity for a ligand than does the oxidized species, the apparent midpoint potential of the redox reaction will be more positive than the formal reduction potential because the coupled reaction lowers the effective concentration of the active reduced species relative to the inactive oxidized species.

[3] Since the activation process involves the free enzyme and not the E−S complex, eq 6 is similar to the equation for competitive inhibition, $(K_m)_{app} = K_m(1 + i/K_i)$.

Scheme 5



the initial velocity and the *V/K* data yielded the same redox potential, only the *V/K* data could be used over a wide range of potentials. To adequately fit the initial velocity data, the window of accessible redox potentials had to be restricted to a single electron acceptor which diminishes the accuracy of the results. Since the *V/K* for one substrate is not a function of the concentration or nature of the other substrate, it was possible to fit the data obtained with four redox mediators. In addition, the *V/K* data could discriminate between the $n = 1$ and $n = 2$ mechanisms better than the initial velocity data. Another advantage to relating *V/K*, instead of initial velocity, to the potential is that, in some cases, the concentrations of the mediators must be adjusted to obtain convenient absorbance changes. In a ping-pong mechanism, *V/K* for one substrate is independent of the concentration of the other substrate.

Thus, one can determine the $E°_e$ for an active site metal center or amino acid residue by measuring the apparent *V/K* at different applied potentials and fitting the data to eq 7. Proteins that fit this classification include metalloenzymes such as hemes and cobamides. Reduction of ferric to ferrous heme increases the affinity for oxygen and other substrates (Sligar, 1976; Walsh *et al.*, 1979; Janick *et al.*, 1983). Reductive activation of the cobalt centers of corrinoid-containing proteins (Harder *et al.*, 1989; Banerjee *et al.*, 1990; Lu *et al.*, 1990, 1994) is required before they can be methylated. The activity of many nonmetal redox enzymes depends on an interconversion between the dithiol and disulfide states. Examples include fructose-1,6-bisphosphatase and NADP-malate dehydrogenase (Crawford *et al.*, 1989) and a valyl-tRNA synthetase (Black, 1983).

Another class of enzymes that can be studied by controlled potential enzymology includes those that perform redox reactions. Examples are numerous and, besides HBCR, include NAD(P)-dependent enzymes, FAD- and FMN-containing proteins, disulfide reductases, heme proteins such as cytochrome P450, and other metalloenzymes such as hydrogenase, carbon monoxide dehydrogenase, and nitrogenase. In this case, the catalytic site cycles between two or more oxidation states during the reaction. Schemes 1−3 and 5 describe some possible mechanisms.

We performed steady-state kinetic experiments at controlled potentials to determine the $E°_e$ of the active site of HBCR. Since the enzyme is present in low amounts, it is difficult to generate enough protein for spectroelectrochemical experiments. However, steady-state kinetics requires much less protein than does spectroscopy. To quantitatively analyze the influence of applied potential on the kinetic parameters of HBCR, it was necessary to define the steady-state mechanism of the enzyme. Our results were consistent with a ping-pong mechanism. Equations 4a, 4b, and 5 were derived to describe the potential dependence of *V/K* for several types of relevant mechanisms. One can distinguish between these mechanisms by comparing the fits of the experimental data to these expressions. Mechanisms involving two-electron reduction of the enzyme before binding of substrate were ruled out. Our results indicated that HBCR is reduced by one electron before substrate binds and that the second electron is delivered subsequently. The $E°_e$ of the active site of HBCR was found to be −0.350 V vs NHE, and maximum values (under optimal redox conditions) for k_{cat} and k_{cat}/K_m were determined.

How can such a mechanism be described for HBCR, which performs a two-electron reduction of the substrate and appears to follow a ping-pong mechanism? A possible explanation is described by Scheme 5. As in a ping-pong mechanism, first, ^{ox}E is reduced, benzyl viologen leaves, and the substrate binds. However, in contrast to a classical ping-pong mechanism, another 1 mol of benzyl viologen binds to ^{red}E before substrate is reduced. Then, two successive one-electron transfers to the substrate occur. In this mechanism, re-reduction of the enzyme by benzyl viologen occurs

in the ternary complex including enzyme, dye mediator, and substrate. There are two limiting cases in Scheme 5. (A) If the first electron transfer to ^{ox}E is slower than the electron transfer within the ternary complex, then Scheme 5 effectively reduces to Scheme 1 and a single titration for a one-electron transfer ($n = 1$) would be observed. The two one-electron transfers are designated **slow** and **fast** in the scheme to correspond to case A. Since the $E°_e$ reflected in the dependence of V_{max}/K_m in eq 4 would correspond to the $^{ox}E/^{red}E$ couple, this mechanism could not be distinguished from a ping-pong mechanism by steady-state kinetics. (B) Conversely, if the transfer of electrons within the ternary complex is slower than to ^{ox}E, then the V_{max}/K_m should show a biphasic dependence on the applied potential. As the applied potential is decreased, the first titration would correspond to the $^{ox}E/^{red}E$ couple and the second to the $^{ox}E/^{red}E$ couple within the ternary complex. In addition, case B would be expected to give the product inhibition patterns for a ternary mechanism. Our results are most consistent with case A because a single $n = 1$ redox titration curve was observed in the controlled potential kinetic studies. In addition, the product inhibition patterns and the fact that *V/K* is independent of the concentration and the chemical nature of the other substrate were inconsistent with case B.

The mechanism for HBCR proposed in Scheme 5 is similar to that described for the Birch reduction, the classical organic chemical reaction for reductive dehydroxylation of phenolic compounds. In the Birch reduction, the intermediate radical anion accumulates. An advantage of the proposed enzymatic mechanism is that radical intermediates do not build up. This is because the substrate is not reduced until the necessary reducing equivalents are in place at the active site (^{red}E and ^{red}BV). This could allow for two rapid successive one-electron transfers to the substrate. This scheme also offers an explanation for why the second electron transfer to the enzyme would be faster than the first. The $^{ox}E-S/^{red}E-S$ complex may have a more positive redox potential than $^{ox}E/^{red}E$. One of many possible mechanisms for such a shift in redox potential is that ligation of the OH group of hydroxybenzoyl-CoA to a metal center should facilitate substrate reduction.

A more complicated situation is represented by enzymes that both require reductive activation and catalyze redox reactions. For example, methyl-CoM reductase performs the two-electron reduction of methyl-CoM to methane. Its Ni^{2+} center requires reduction to the 1^+ state before methyl-CoM can be reduced (Rouviére & Wolfe, 1988). Hydrogenase requires reductive activation and also performs the two-electron reduction of protons to H_2 (Fernandez *et al.*, 1984). Both the copper (Pember *et al.*, 1986) and the non-heme-iron-containing phenylalanine hydroxylases are activated by one-electron reductions, and the active sites appear to undergo redox changes during their reactions. In these cases, *V/K* can be more complicated since the site that requires activation may not be the same site that cycles during the reaction.

In order to ascertain the generality of the phenomenon and the kinetic treatment described, the influence of applied potential on the kinetic parameters of a number of enzymes should be examined. Such a treatment will likely lead to much higher catalytic activities of some of these enzymes. This is because most enzyme assays are performed in an aerobic environment in the absence of reducing agents. Under these conditions, the oxygen concentration is 0.245 mM and the ambient potential is 0.81 V (Hungate, 1969). The cytoplasm of *Escherichia coli* cells growing aerobically has been estimated to be between −0.280 and −0.42 V (Taylor *et al.*, 1990). Assuming that the ambient potential of most growing cells is not vastly different from that of *E. coli*, then the ambient potential of the assay mixture is approximately 1 V more positive than that in growing cells. If the $E°_e$ for the active site of an enzyme requires a one-electron reductive activation in the physiological range (for the sake of argument, −200 mV), then the measured activity in a standard assay mixture would be $2 \times 10^{-15}\%$ ($10^{-(1000/60)}$) of the activity measured at an applied potential equal to the $E°_e$. This would be equivalent to performing an enzyme reaction 17 pH units away from the pK_a.

APPENDIX

Theory

Dependence of V/K on potential for a Reductively Activated Enzyme. In the equations described below, O and R represent the oxidized and reduced dye mediators. The redox part of the scheme for a reductively activated enzyme that does not catalyze a redox reaction can be described by Scheme 4. One can derive eq 8 (eq 6, above):

$$v = \frac{X_{max}S}{K_m\left(1 + \frac{^{ox}E}{^{red}E}\right) + S} \tag{8}$$

$^{ox}E/^{red}E$ can be related to the reduction potential for the enzyme by the Nernst relationship (eq 9):

$$E = E°_e + \frac{RT}{nF}\ln\frac{^{ox}E}{^{red}E} \tag{9a}$$

Therefore,

$$\frac{^{ox}E}{^{red}E} = \exp\left[\frac{nF}{RT}(E - E°_e)\right] \tag{9b}$$

Substituting into eq 8 yields eq 10:

$$v = \frac{V_{max}S}{K_m\left\{1 + \exp\left[\frac{nF}{RT}(E - E°_e)\right]\right\} + S} \tag{10}$$

One then can relate the *V/K* directly to the redox potential for the enzyme:

$$(V/K_m)_{app} = \frac{V_{max}}{K_m\left\{1 + \exp\left[\frac{nF}{RT}(E - E°_e)\right]\right\}} \tag{11}$$

For a one-electron reductive activation process at 30 °C, one can attain eq 12:

$$(V/K_m)_{app} = \frac{V_{max}}{K_m\{1 + 10^{[(E-E°_e)/60]}\}} \tag{12}$$

Dependence of V/K on Potential for a Ping-pong Oxidation−Reduction Reaction. The reaction of a redox-activated enzyme catalyzing a reaction in a ping-pong-type mechanism

can be modeled by Scheme 1. Using the transit times procedure and taking into account all the microscopic rate constants, one can derive the rate law (eq 13) for the reaction:

$$\frac{E_t}{v} = \frac{1}{k_1[R]} + \frac{k_2}{k_1[R]k_3} + \frac{k_2k_4}{k_1[R]k_3k_5} + \frac{k_2k_4k_6[O]}{k_1[R]k_3k_5k_7[S]} + \frac{k_2k_4k_6[O]k_8}{k_1[R]k_3k_5k_7[S]k_9} + \frac{k_2k_4k_6[O]k_8k_{10}}{k_1[R]k_3k_5k_7[S]k_9k_{11}} + \frac{1}{k_3} + \frac{k_4}{k_3k_5} + \frac{k_4k_6[O]}{k_3k_5k_7[S]} + \frac{k_4k_6[O]k_8}{k_3k_5k_7[S]k_9} + \frac{k_4k_6[O]k_8k_{10}}{k_3k_5k_7[S]k_9k_{11}} + \frac{1}{k_5} + \frac{k_6[O]}{k_5k_7[S]} + \frac{k_6[O]k_8}{k_5k_7[S]k_9} + \frac{k_6[O]k_8k_{10}}{k_5k_7[S]k_9k_{11}} + \frac{1}{k_7[S]} + \frac{k_8}{k_7[S]k_9} + \frac{1}{k_9} + \frac{k_{10}}{k_9k_{11}} + \frac{k_8k_{10}}{k_7[S]k_9k_{11}} + \frac{1}{k_{11}} \quad (13)$$

Rearranging in a Michaelis–Menten form:

$$\frac{E_t}{v} = \frac{1}{[R]}K_m^{\,r}\frac{1}{k_{cat}} + \frac{1}{[S]}K_m^{\,s}\frac{1}{k_{cat}} + \frac{1}{k_{cat}} + \frac{[O]}{[S]}k_m^{\,s}\frac{1}{k_{cat}k_5}k_6\left(1 + \frac{k_4}{k_3} + \frac{k_2k_4}{[R]k_1k_3}\right) \quad (14)$$

where

$$\frac{K_m^{\,r}}{k_{cat}} = \frac{1}{k_1} + \frac{k_2}{k_1k_3} + \frac{k_2k_4}{k_1k_3k_5}$$

$$\frac{K_m^{\,s}}{k_{cat}} = \frac{1}{k_7} + \frac{k_8}{k_7k_9} + \frac{k_8k_{10}}{k_7k_9k_{11}}$$

$$\frac{1}{k_{cat}} = \frac{1}{k_3} + \frac{1}{k_5} + \frac{k_4}{k_3k_5} + \frac{1}{k_9} + \frac{1}{k_{11}} + \frac{k_{10}}{k_9k_{11}}$$

$$\frac{v}{E_t} = \frac{k_{cat}[R][S]}{K_m^{\,r}[S] + [R][S] + k_m^{\,s}[R]\left\{1 + \frac{[O]}{K_i^{\,O}}\left(1 + \frac{k_4}{k_3} + \frac{K_i^{\,r}k_4}{[R]k_3}\right)\right\}} \quad (15)$$

$$K_i^{\,O} = \frac{k_5}{k_6} \quad \text{and} \quad K_i^{\,r} = \frac{k_2}{k_1}$$

Then,

$$v = \frac{E_tk_{cat}[R][S]}{(K_m^{\,r} + [R])[S] + K_m^{\,s}[R]\left\{1 + \frac{[O]}{K'_i{}^{O}} + \left(\frac{[O]K'_i{}^{r}}{[R]K'_i{}^{O}}\right)\right\}} \quad (16)$$

$$K'_i{}^{O} = \frac{K_i^{\,O}}{1 + \frac{k_4}{k_3}} \quad \text{and} \quad K'_i{}^{r} = K_i^{\,r}\frac{k_4}{k_3}$$

From eq 16, one can determine that

$$\frac{V_{max}}{K_m} = \frac{E_tk_{cat}}{K_m^{\,s}\left\{1 + \frac{[O]}{K'_i{}^{O}} + \left(\frac{[O]K'_i{}^{r}}{[R]K_i^{\,O}}\right)\right\}} \quad (17)$$

In order to introduce the Nernst equation into eq 17, consider the cell reaction:

$$^{ox}E + BV^{\bullet+} \rightleftharpoons {}^{red}E + BV^{2+}$$

The equilibrium constant obeys the following relationship:

$$K_{eq} = \frac{k_1k_3k_5}{k_2k_4k_6} = \frac{K_i^{\,O}}{K'_i{}^{r}} \quad (18)$$

and is related to the free energy and the ambient potential

$$\Delta G^\circ = nFE^\circ = -RT\ln K_{eq} \quad (19)$$

The E° of the reaction is written as a function of the redox potentials of the enzyme and the dye:

$$E^\circ = E^\circ{}_e - E^\circ{}_d \quad (20)$$

Substituting, yields eq 21.

$$K_{eq} = e^{\frac{nF}{RT}(E^\circ{}_e - E^\circ{}_d)} \quad (21)$$

The Nernst equation is

$$E = E^\circ + \frac{RT}{nF}\ln\frac{[O]}{[R]} \quad (22)$$

Combining (17), (18), (21), and (22) yields eq 23:

$$\frac{V}{K} = \frac{\left(\frac{V}{K}\right)\max}{1 + \frac{[O]}{K'_i{}^{O}} + e^{\frac{nF}{RT}(E - E^\circ{}_e)}} \quad (23)$$

Equation 23 gives a general relationship between the applied potential E and the kinetic parameter V_{max}/K_m of the enzymatic reaction and allows one to measure the formal reduction potential of the active site of the enzyme, $E^\circ{}_e$.

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