Mechanism of Ascorbic Acid Oxidation by Cytochrome b\textsubscript{561}

David Njus,* Michael Wigle,‡ Patrick M. Kelley,† Brian H. Kipp,‡,∥ and H. Bernhard Schlegel§

Departments of Biological Sciences and Chemistry, Wayne State University, Detroit, Michigan 48202

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ABSTRACT: The 1 equiv reaction between ascorbic acid and cytochrome b\textsubscript{561} is a good model for redox reactions between metalloproteins (electron carriers) and specific organic substrates (hydrogen-atom carriers). Diethyl pyrocarbonate inhibits the reaction of cytochrome b\textsubscript{561} with ascorbate by modifying a histidine residue in the ascorbate-binding site. Ferri/ferrocyanide can mediate reduction of DEPC-treated cytochrome b\textsubscript{561} by ascorbic acid, indicating that DEPC-inhibited cytochrome b\textsubscript{561} cannot accept electrons from a hydrogen-atom donor like ascorbate but can still accept electrons from an electron donor like ferrocyanide. Ascorbic acid reduces cytochrome b\textsubscript{561} with a $K_m$ of 1.0 ± 0.2 mM and a $V_{max}$ of 4.1 ± 0.8 s\textsuperscript{-1} at pH 7.0. $V_{max}/K_m$ decreases at low pH but is approximately constant at pH > 7. The rate constant for oxidation of cytochrome b\textsubscript{561} by semidehydroascorbate decreases at high pH but is approximately constant at pH < 7. This suggests that the active site must be unprotonated to react with ascorbate and protonated to react with semidehydroascorbate. Molecular modeling calculations show that hydrogen bonding between the 2-hydroxyl of ascorbate and imidazole stabilizes the ascorbate radical relative to the monoanion. These results are consistent with the following mechanism for ascorbate oxidation. (1) The ascorbate monoanion binds to an unprotonated site (histidine) on cytochrome b\textsubscript{561}. (2) This complex donates an electron to reduce the heme. (3) The semidehydroascorbate anion dissociates from the cytochrome, leaving a proton associated with the binding site. (4) The binding site is deprotonated to complete the cycle. In this mechanism, an essential role of the cytochrome is to bind the ascorbate monoanion, which does not react by outer-sphere electron transfer in solution, and complex it in such a way that the complex acts as an electron donor. Thermodynamic considerations show that no steps in this process involve large changes in free energy, so the mechanism is reversible and capable of fulfilling the cytochrome’s function of equilibrating ascorbate and semidehydroascorbate.

In enzymatic redox reactions, a metalloprotein typically catalyzes the transfer of reducing equivalents to and from organic compounds. Because the organic substrates gain and lose hydrogen atoms while the metal transfers electrons, the enzyme must move protons to and from the substrate coincident with electron transfer. Elucidating the enzymatic mechanisms catalyzing this concerted proton—electron transfer is thus of fundamental significance to understanding biological redox reactions.

Potentially simple examples of concerted proton—electron transfer are reactions of ascorbic acid because these reactions transfer only a single reducing equivalent. When it oxidizes, ascorbic acid loses the equivalent of a single hydrogen atom (1); the ascorbate monoanion, the predominant form at physiological pH, is oxidized to the radical anion, semidehydroascorbate. The enzyme’s metal center accepts an electron from ascorbic acid, leaving a proton to be deposited elsewhere.

The mechanism by which this occurs may be examined in cytochrome b\textsubscript{561}, a membrane-bound protein that maintains ascorbate concentrations within secretory vesicles by equilibrating intravesicular and cytosolic pools of ascorbic acid and semidehydroascorbate (2–6). Because cytochrome b\textsubscript{561} reacts only with ascorbic acid and semidehydroascorbate, the reaction mechanism is not complicated by coupling to other redox reactions. We hypothesize that the ascorbate monoanion binds to an unprotonated histidine residue on the cytochrome. The complexed ascorbate donates an electron. The product then dissociates as the radical anion, semidehydroascorbate, leaving the proton on the histidine residue. We have used kinetic, molecular modeling, and thermodynamic considerations to examine this hypothesis.

EXPERIMENTAL PROCEDURES

Chromaffin vesicles were isolated from bovine adrenal medulla. Medullae excised from fresh bovine adrenals were homogenized in isolation medium [0.3 M sucrose and 10 mM Hepes (NaOH) at pH 7.0]. The homogenate was centrifuged at 750g for 15 min at 4 °C, and the supernatant was then centrifuged at 27000g for 20 min. After resuspension in isolation medium, the pellet was centrifuged and resuspended again in the isolation medium. The vesicles were

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* Abbreviations: DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; FeCy, ferrocyanide; Hepes, N-((2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid; Mops, 3-(N-morpholino)propane-sulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; SD, standard deviation; Tris, tris(hydroxymethyl)aminomethane.
DEPC reaction was terminated by adding histidine (18 mM final concentration). Diethyl pyrocarbonate (13.8 mM final concentration) was added to half of the membrane suspension. After the DEPC reaction, vesicle membranes were thawed and dialyzed for 24 h at 4 °C. For studies of reaction kinetics (Figure 2), chromaffin-vesicle membranes were thawed and centrifuged at 27000g for 20 min at 4 °C. The pellet was resuspended in dialysis medium [0.15 M KCl, 10 mM EDTA, and 10 mM Mops (pH 7.0)] and dialyzed for 48 h at 4 °C against dialysis medium (4 × 100 volumes) to remove residual ascorbic acid and catecholamines. These chromaffin-vesicle ghosts were then purified on a Ficoll/sucrose density gradient.

Cytochrome $b_{561}$ reduction was followed spectrophotometrically using an Aminco-Morrow stopped-flow apparatus equipped with an SLM-Aminco DW2000 spectrophotometer operated in the dual-wavelength mode. Ascorbic acid was freshly prepared in 0.2 M KCl, 10 mM methylimidazole, 200 μM EDTA, and 10 mM Hepes (pH 7.0, 7.5, or 8.0). This was mixed with ghosts diluted into the same medium, and the absorbance difference (561 nm – 569 nm) was recorded. The initial rate of cytochrome $b_{561}$ reduction was converted to units of inverse seconds using a molar extinction coefficient of 17 500 M$^{-1}$ cm$^{-1}$.

The Michaelis–Menten equation:

$$V = \frac{V_{\text{max}}[\text{AH}^-]}{([\text{AH}^-] + K_m)}$$

For studies in D$_2$O (Figure 6), cytochrome $b_{561}$ was solubilized in 0.1% NP40 after the membranes had been washed in 1% Tween 20 as described by Wakefield et al. (11). Solubilized cytochrome $b_{561}$ was then dialyzed against 200 mM NaCl, 20 mM Hepes, and 1 mM EDTA (pH 6.8) in either D$_2$O or H$_2$O. The cytochrome was mixed by a stopped-flow method with ascorbic acid in the same medium, and the initial rate of reduction (0.67 μM cytochrome $b_{561}$) was followed spectrophotometrically as described above.

Molecular modeling calculations were carried out with the Gaussian series of programs (12). To simplify the computations, the side chain of ascorbate was removed, histidine was modeled by imidazole, and the geometry optimization was restricted to structures in which the ascorbate and imidazole rings were coplanar. Note that the actual geometry need not be coplanar, because rotation about the hydrogen bond to

![Figure 1: DEPC inhibits reduction of cytochrome $b_{561}$ by ascorbate but not by ferrocyanide. Chromaffin-vesicle membranes were divided; half were treated with DEPC (A and B), and the rest were kept as an untreated control (C and D). Reduction of cytochrome $b_{561}$ in 1 mL samples was monitored spectrophotometrically. Ascorbate (50 μL of a 100 mM solution) and ferrocyanide (50 μL of a 100 mM solution) were added at the indicated times. Finally, a few grains of sodium dithionite were added to determine 100% cytochrome reduction.](image1)

![Figure 2: Dependence of the cytochrome $b_{561}$ reduction rate on ascorbate concentration. Chromaffin-vesicle membranes were mixed with ascorbic acid by a stopped-flow method, and the absorbance of cytochrome $b_{561}$ was recorded as described in Experimental Procedures. Each point is the average (±SD) of three separate determinations of the initial rate of reduction at pH 5.5 (○) or 8.0 (●). The lines are least-squares fits to eq 1.](image2)
any arbitrary angle is not expected to have a significant effect on energy. Geometries of the monomers and hydrogen-bonded dimers were optimized at the HF/6-31G* level of theory. Relative energies of ascorbate in different oxidation and protonation states were calculated at the PMP2/6-31+G** and B3LYP/6-31+G** levels of theory using the HF/6-31G*-optimized geometry. Test calculations on hydrogen bonding between acetic acid, acetate, and imidazole indicated that the HF/6-31G* interaction energies were within 1–2 kcal/mol of the PMP2/6-31+G** and B3LYP/6-31+G** values. Hence, the hydrogen bond energies for ascorbate were calculated at the HF/6-31G* level of theory.

Protein concentrations were determined using the BCA assay (13). DEPC, Hapes, Mes, Mops, and Tris were obtained from Sigma Chemical Co.

RESULTS

Although cytochrome b<sub>561</sub> reacts with ascorbate on both sides of the chromaffin–vesicle membrane, reaction is normally observed only at the external site because of spatial and kinetic factors. Externally added ascorbate does not permeate to the inside of resealed chromaffin–vesicle ghosts (14). Moreover, cytochrome b<sub>561</sub> is reduced 1 order of magnitude faster from the outside than from the inside (15), so any ascorbate penetrating into the ghosts would cause only a negligible fraction of the total observed reduction.

Cytochrome b<sub>561</sub> has an essential histidine residue in the external ascorbate-binding site (16). Ethoxyformylation of this histidine residue with diethyl pyrocarbonate prevents ascorbate from directly reducing cytochrome b<sub>561</sub>, but allows ferricyanide to mediate the transfer of electrons from ascorbate to the cytochrome's heme (Figure 1). Although ferricyanide addition oxidizes the cytochrome, the presence of ferricyanide accelerates the reduction of cytochrome b<sub>561</sub> by ascorbate in DEPC-treated membranes (Figure 1A,B). In untreated membranes, ascorbate reduces cytochrome b<sub>561</sub> quickly in the presence or absence of ferricyanide (Figure 1C,D). This argues that the histidine residue is required for reaction with the hydrogen-atom donor ascorbate but is not needed for reaction with the electron donor ferrocyanide.

To test the possibility that the histidine residue functions in proton transfer, the pH dependence of the reaction between ascorbic acid and cytochrome b<sub>561</sub> was investigated. The rate of cytochrome b<sub>561</sub> reduction follows Michaelis–Menten kinetics, saturating at high concentrations of external ascorbate (Figure 2). K<sub>m</sub> is much higher at pH 5.5 than at pH 8.0, although the V<sub>max</sub> values are comparable. At pH 7.0, ascorbate reduces cytochrome b<sub>561</sub> with a K<sub>m</sub> of 1.0 ± 0.2 mM and a V<sub>max</sub> of 4.1 ± 0.8 s<sup>−1</sup>. This compares to a K<sub>m</sub> of 0.34 mM reported by Flatmark and Terland (17).

Over the physiological pH range, V<sub>max</sub> changes little (Figure 3A), while K<sub>m</sub> increases markedly at low pH (Figure 3B). As a consequence, a plot of V<sub>max</sub>/K<sub>m</sub> is constant above and decreases below pH 6.5 (Figure 4). Because ascorbate does not have a p<sub>K</sub> near 7, the kinetics are consistent with ascorbate binding to an unprotonated histidine residue on the cytochrome.

The reverse reaction, oxidation of cytochrome b<sub>561</sub> by semidehydroascorbate, may also be examined. It is not practical to increase the ascorbate radical concentration to a saturating level, but we have been able to determine an apparent rate constant, an approximation to V<sub>max</sub>/K<sub>m</sub> (18). This rate constant diminishes at pH >6.5 (Figure 4), suggesting that the ascorbate radical reacts with the protonated histidine.

Other potential sources of pH dependence may be excluded. The pK<sub>s</sub> of ascorbic acid (pK<sub>1</sub> = 4.5, pK<sub>2</sub> = 11.34) and its radical (pK<sub>r</sub> = −0.45) lie outside of the physiological range. The midpoint reduction potential of the heme is independent of pH (19).

To see how a histidine residue might interact with ascorbic acid in the cytochrome’s binding site, we have used a
molecular modeling approach. The effect of hydrogen bonding between histidine and ascorbate on the stability of the various ascorbate forms may be assessed by calculating hydrogen bonding energies. To simplify the calculations, unprotonated imidazole was used to represent histidine and an ascorbate analogue lacking the side chain was used to represent ascorbate (Figure 5). Molecular modeling is limited to simple systems and does not take into account effects of solvent or neighboring groups. As a consequence, absolute values of energies are not significant, but relative energies can help define which structures are most likely.

Molecular modeling calculations show several things. When imidazole and the dianion of the ascorbate analogue are allowed to interact, energy minimization results in the transfer of a proton from the imidazole to the dianion, yielding a complex between the monoanions of imidazole and the ascorbate analogue. This argues again that the ascorbate dianion cannot be stabilized by the active site of the cytochrome and, therefore, the monoanion is more likely to be the electron donor to the heme.

The monoanion can exist in two possible forms depending on which of the two hydroxyl groups ionizes. The more stable form in solution has the undissociated proton on the 2-hydroxyl group. This structure permits resonance, distributing the negative charge between the oxygen atoms on C1 and C3. Molecular modeling confirms that this is the more stable form (Figure 5).

The ascorbate monoanion could interact with imidazole through hydrogen bonds at any one of the three oxygen atoms on C1, C2, or C3. Hydrogen bonding through the 2-hydroxyl of the ascorbate analogue stabilizes the radical relative to the monoanion. This is not the case for hydrogen bonding through the oxygen atoms at the C1 or C3 position, however. Hydrogen bonding through the 2-hydroxyl group reduces the influence of that H and allows the unpaired electron to be distributed over three oxygen atoms. By contrast, hydrogen bonding at either of the other two positions leaves this H in place and introduces another H from the imidazole, thus further constraining the distribution of the unpaired electron.

Finally, energy minimization shows that atomic coordinates do not change significantly when the ascorbate analogue, hydrogen bonded to imidazole through the 2-hydroxyl group, is oxidized to the free radical. This means that electron transfer from the bound ascorbate monoanion may occur without requiring molecular reorientation.

Since the ascorbate monoanion loses a proton along with an electron when reducing cytochrome $b_{561}$, the reaction might exhibit a deuterium isotope effect. Indeed, substituting D2O for H2O slows the rate at which ascorbate reduces cytochrome $b_{561}$ to between 14 and 40% of the rate in H2O (Figure 6). The effect is greatest at low ascorbate concentrations, indicating that $K_m$ increases slightly in D2O and $V_{max}$ is diminished considerably. By contrast, the rate at which ascorbate reduces cytochrome $c$ is changed little in D2O (data not shown).

**DISCUSSION**

A synthesis of the data presented here along with other information leads to a hypothesis for the mechanism of cytochrome $b_{561}$ reduction by ascorbic acid (Figure 7). Central features of this model are as follows. (1) The ascorbate monoanion binds to an unprotonated site (histidine) on cytochrome $b_{561}$. (2) This complex donates an electron to reduce the heme. (3) Semidehydroascorbate dissociates from the cytochrome as the radical anion, transferring a proton to the binding site. (4) The binding site is deprotonated to complete the cycle. Let us consider the evidence for this mechanism beginning with the ascorbate-binding site.

Chemical modification studies (16, 20) using diethyl pyrocarbonate suggest that the ascorbate-binding site contains...
These observations argue, therefore, that DEPC modification interferes specifically with the reaction of cytochrome b$_{561}$ with ascorbic acid and does not affect the heme. Moreover, ascorbic acid protects against inactivation of the cytochrome by DEPC (16, 20), suggesting that DEPC reacts in the ascorbate-binding site. DEPC reacts with histidine much more slowly at acidic pH, because it ethoxylformylates the unprotonated imidazole. The rate of cytochrome b$_{561}$ inactivation by DEPC slows at pH <7, consistent with modification of a histidine residue (16). Finally, Tsubaki et al. (21) have used mass spectrometry to identify the residues modified by DEPC as Lys85, His161, and either His88 or His92. The three histidine residues are all on the external side of the membrane; one of them is likely to be the essential histidine residue in the ascorbate-binding site.

Ascorbic acid exists in undissociated, monoanionic, and dianionic forms where pK$_1$ = 4.5 and pK$_2$ = 11.34. The ascorbate dianion is a potent electron donor in solution, but several observations make it unlikely that the bound dianion is actually the electron donor to the heme. First, the dianion is present in an exceedingly low concentration at physiological pH. Second, the dianion concentration will increase 10-fold with a 1 unit increase in pH, but the pH dependence of cytochrome b$_{561}$ reduction is not nearly this strong, at least at pH >7 (Figures 3 and 4). Finally, molecular modeling suggests that a histidine residue in the binding site will not stabilize the dianion but will instead donate a proton to it.

The pH dependencies of K$_m$ and V$_{max}$ are consistent with the ascorbate monoanion binding and then transferring an electron to the heme. To analyze this quantitatively, consider the mechanism shown in Figure 7. To analyze this model, we assume that the electron transfer step is slow relative to the binding steps, so the binding and protonation reactions are effective at equilibrium and are adequately described by the constants K$_{Dm}$ (monoanion binding), K$_{Dr}$ (radical binding), and K$_c$ (histidine protonation). Then, if a steady state is assumed, K$_m$ and V$_{max}$ are as follows:

$$V_{max} = k_{red}$$

(2)

$$K_m = K_{Dm}(1 + [H^+]/K_c)$$

(3)

$$V_{max}/K_m = k_{red}[K_{Dm}(1 + [H^+]/K_c)]$$

(4)

where $k_{red}$ is the rate constant for electron transfer from bound ascorbate to the heme.

The data for K$_m$ (Figure 3B) and V$_{max}$/K$_m$ (Figure 4) are consistent with these equations assuming a value of $\sim$6.5 for pK$_c$. This supports the idea that the ascorbate monoanion binds to an unprotonated site with a pK near neutrality. This is compatible with the presence of a histidine residue in the ascorbate-binding site of cytochrome b$_{561}$. The fact that D$_2$O slows cytochrome b$_{561}$ reduction largely by reducing V$_{max}$ (Figure 6) argues that D$_2$O acts primarily on $k_{red}$, the rate constant for the electron transfer step itself. This is consistent with a proton playing a key role in the redox reaction mechanism.

There have been suggestions that cytochrome b$_{561}$ contains two hemes arranged in series to conduct electrons across the membrane following the paradigm of mitochondrial cytochrome b$_{561}$/b$_{561}$ (22–25). A second heme would not affect the kinetic analysis presented here because the measurement
of initial rates limits the data to reduction of the first heme. Rapid electron transfer between two hemes would not affect the quantitation of reduction, because the hemes have indistinguishable absorption spectra \(19\).

According to the mechanism described above, the bound ascorbate monooanion acts as an electron donor and forms the bound semidehydroascorbate radical as an intermediate. The monooanion in solution is a relatively poor electron donor having a reduction potential of 0.766 V. If it is to reduce cytochrome \(b_{561} (E^0 = 0.140 \text{ V})\), then the ascorbate-binding site must destabilize the monooanion and stabilize the free radical to facilitate the electron transfer reaction. Molecular modeling shows that hydrogen bonding with the imidazole may accomplish this if the unprotonated imidazole is hydrogen bonded to the 2-hydroxyl of the ascorbate monoanion. The hydrogen bond energy at this location is relatively stronger for the free radical than for the monooanion. Hydrogen bond energies at the other two oxygen atoms are stronger for the monooanion than for the free radical. Furthermore, minimized structures of imidazole--ascorbate complexes hydrogen bonded through \(C_2\) show that the various forms of ascorbate (monooanion, free radical, and radical anion) all have similar atomic coordinates. Thus, reorientation of the ascorbate--histidine complex does not need to occur as the cytochrome proceeds through the reduction reaction.

The bound ascorbate free radical must dissociate from the site and lose a proton to form the semidehydroascorbate radical anion. This could happen by (1) the bound radical dissociating and then deprotonating, (2) the bound radical losing a proton and then dissociating as the semidehydroascorbate radical anion, or (3) the bound radical transferring a proton to the binding site and then dissociating as semidehydroascorbate. The third case is consistent with the kinetics of oxidation by semidehydroascorbate (Figure 4).

The \(pH\) dependence of the rate constant suggests that the semidehydroascorbate radical anion reacts with a protonated site having a \(pK\) of ~6.5. The other two possibilities are energetically improbable. In the first case, stabilization of the bound radical sufficient to achieve a reasonable reduction potential for electron donation (0.3 V) would require an extremely tight binding affinity \(K_D = 10^{-11} \text{ M}\). In the second case, either the semidehydroascorbate would have to bind with very high affinity or the \(pK\) of the bound radical would have to increase substantially. To achieve a dissociation constant of 1 \(\mu\text{M}\) with a reasonable reduction potential, the \(pK\) would have to increase to 5 from the value of ~0.45 observed in solution.

The third case, by contrast, can be achieved with reasonable energetics. Thermodynamic equilibrium in this system (Figure 7) relates the parameters \(K_{Dm}, E^0_c, K_{Dr}, pK_c, pK_r, \text{ and } E^0_r\):

\[
E^0_c = E^0_r + (RT/F \log e)(\log K_{Dr} - \log K_{Dm} - pK_c + pK_r)
\]

Knowing \(E^0_r = 0.766 \text{ V}, K_{Dm} = 1 \text{ mM}, \text{ and } pK_c = -0.45\), we can determine \(E^0_c\) as a function of \(K_{Dr}\) and \(pK_c\) (Figure 8). \(pK_c\), the \(pK\) of the active site residue, is ~6 or ~7. \(K_{Dr}\), the binding constant for semidehydroascorbate, is unknown, but in studies of cytochrome \(b_{561}\) oxidation, we could not observe saturation by semidehydroascorbate up to concentra-

\[E^0_c = E^0_r + (RT/F \log e)(\log K_{Dr} - \log K_{Dm} - pK_c + pK_r)
\]

This is consistent with a midpoint potential of the bound ascorbate or semidehydroascorbate being ~300 mV, 160 mV more positive than the midpoint potential of the cytochrome (140 mV; 17, 19). A midpoint potential of this magnitude would be compatible with a dissociation constant for semidehydroascorbate \(K_{Dr}\) of ~100 \(\mu\text{M}\) (Figure 8). These values not only are reasonable but also show that the mechanism outlined in Figure 7 does not involve any steps requiring large changes in free energy. The reactions, therefore, are all readily reversible, a necessity for a cytochrome whose function is to equilibrate ascorbate and semidehydroascorbate.

The mechanism proposed here hypothesizes that the reactivity of ascorbate is controlled by the ascorbate-binding site. The heme serves only as an acceptor in the electron transfer reaction. As a consequence, ascorbate bound to cytochrome \(b_{561}\) is poised for electron donation to any suitable electron acceptor. If the heme is already reduced, then the bound ascorbate may react adventitiously with another oxidant. Of particular interest is molecular oxygen, which may be reduced to the superoxide radical anion, initiating the production of a variety of damaging oxygen radicals. The reduction potential of the \(O_2/O_2^+\) pair is ~300 mV under physiological conditions (1, 26). To avoid generation of additional superoxide, it is obviously advantageous for the bound ascorbate to have a midpoint potential that is ~300 mV, implying a binding constant for semidehydroascorbate that is 100 \(\mu\text{M}\) or weaker (Figure 8). As discussed above, these are likely values.

According to the Marcus theory for electron transfer reactions in solution (27), outer-sphere electron transfer reactions are spontaneous and occur at rates that depend solely on the intrinsic reactivity of the compounds involved and on the difference in their relative reduction potentials.

Such reactions are obviously not well suited to biological systems in which reaction rates are carefully regulated and desired redox reactions often involve reactants with relatively
close reduction potentials as, for example, in respiratory and other redox chains. The mechanism presented here suggests that biological systems have brought redox reactions under control by selecting redox compounds that do not react by outer-sphere electron transfer but can be made to behave as electron donors and acceptors when bound in an appropriate site. The function of the redox enzyme, therefore, is to provide both a site for unlocking the electron-transferring capability of the substrate and also a redox center with which the bound substrate can react, preempting spontaneous reactions with other undesired reactants. An attraction of this perspective is that, like the Marcus theory for uncatalyzed reactions, it allows enzymatic redox reactions to be analyzed, at least to a first approximation, in terms of the separate properties of the reactants: a complexed organic substrate and the enzyme’s metal center.

REFERENCES
