Inhibition of Intramolecular Electron Transfer in Sulfite Oxidase by Anion Binding

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Received March 20, 1992

Electron-transfer (ET) rates in proteins and enzymes are often difficult to predict or interpret using the theory developed by Marcus and others, which includes factors such as intercenter distance, intervening medium, orientational effects, and thermodynamic driving force.[3] Further complication derives from the possibility that the observed ET rate may be controlled by the rate of some other process, such as a conformational change.[4] In the present communication, we provide direct kinetic evidence that anion binding to sulfite oxidase limits the rate of intramolecular ET between the redox centers of this enzyme.

Sulfite oxidase isolated from chicken liver is a dimeric protein composed of two identical subunits of 51 550 Da, each containing a pterin-molybdenum cofactor[5] at the catalytic site and a *b*-type heme, in a separate domain which is similar in sequence to cytochrome b_5.[6-8] The enzyme oxidizes sulfite to sulfate, reducing 2 equiv of cytochrome *c* (eq 1). During catalysis electrons flow

$$SO_3^{2-} + 2Fe^{III}\ \text{cyt}\ c \rightleftharpoons SO_4^{2-} + 2Fe^{II}\ \text{cyt}\ c \qquad (1)$$

from sulfite to the molybdenum center to the heme group and then to cytochrome *c*.[9] Steady-state kinetic experiments have shown that anions such as SO_4^{2-}, Cl^-, and HPO_4^{2-} are inhibitors of the flow of electrons from sulfite to cytochrome *c* but not to O_2.[6,10] In 1971, Cohen and Fridovich proposed (regarding inhibition by sulfate) that "the sulfate sensitive step was not the reduction of the enzyme by sulfite, but was rather the egress of electrons from the enzyme to the 1-electron acceptors".[6b] Changes in EPR[10,11] and X-ray absorption[12] spectra have demonstrated that anion binding can occur at the molybdenum center.

Microcoulometric titrations of sulfite oxidase suggest that both the magnitude and the direction of the driving force for intramolecular ET between the molybdenum and iron centers of sulfite oxidase can be modulated by pH and anion concentration.[13] At low pH and/or high chloride, the Mo(VI) center appears to be reduced more easily than the Fe(III) center of the *b*-type heme, whereas at high pH and/or low chloride the Fe(III) center is calculated to be more easily reduced.[13]

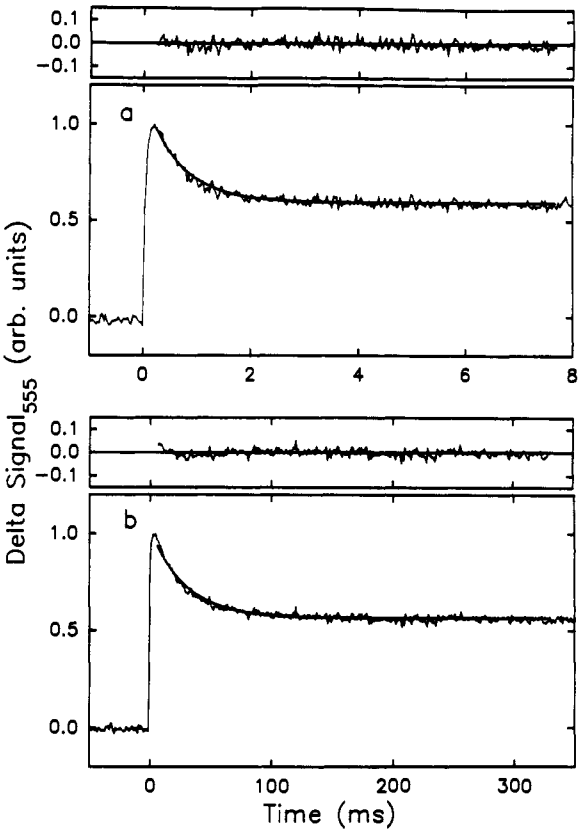

Figure 1. Kinetic traces obtained at 555 nm illustrating heme photoreduction and reoxidation upon laser flash photolysis of sulfite oxidase (50 μM), using 5-deazariboflavin (90 μM) as a reductant: (a) 0 added sulfate, 15 ms time scale, k_{obsd} for heme reoxidation 1580 s^{-1}; (b) 23 mM sulfate, 500 ms time scale, k_{obsd} = 35 s^{-1}. The buffer composition is 6 mM Tris, 6 mM Bis-Tris, 6 mM Bis-tris propane,[19] and 5 mM EDTA adjusted to pH 6 with acetic acid. The laser flash generates <1 μM deazariboflavin semiquinone; thus only a single electron enters each protein molecule. The solid line indicates a single exponential fit to the reoxidation kinetics, and the residual is shown above the trace.

Laser photoreduced flavins provide a powerful method for investigating intramolecular ET in proteins in the absence of the substrate and the terminal electron acceptor of the complete catalytic reaction system.[14,15] Using 5-deazaflavin semiquinone radicals at pH 6 in low ionic strength buffer, the initial rapid second-order reduction of the heme center ($k = 4 \times 10^8$ M^{-1} s^{-1}) is followed by a heme reoxidation reaction that is enzyme concentration independent and that has a rate constant of 1580 s^{-1} (Figure 1a). This heme reoxidation is consistent with ET from Fe(II) to Mo(VI). Note that the net direction of ET is opposite that occurring during enzyme turnover and that the reoxidation of the heme is not complete, implying an equilibrium between the sites. Titration of the sample with sodium sulfate or sodium chloride dramatically decreases the observed rate constant of intramolecular ET from 1580 to <100 s^{-1} (Figure 1b). The extent of heme reoxidation remains constant throughout the titrations, indicating that the redox potentials of the two sites do not change. Kinetic transients for heme reoxidation obtained at all anion

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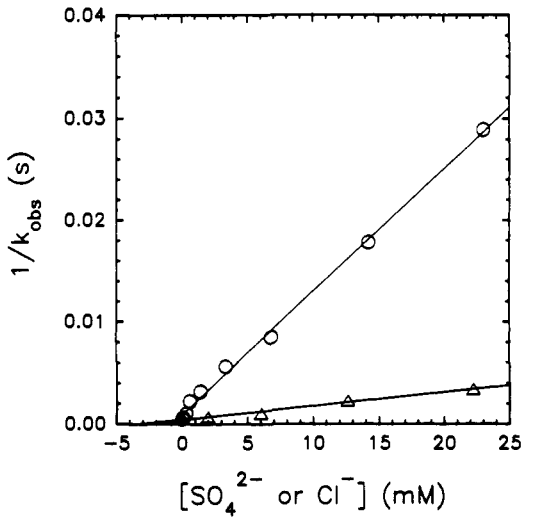

Figure 2. Plot of 1/observed rate constant (heme reoxidation) vs [I] for I = SO_4^{2-} (○) and Cl^- (△). Experiments were performed under the conditions described in Figure 1. Dissociation constants K_i are 0.8 (±0.2) mM for SO_4^{2-} and 4 (±1) mM for Cl^- and can be obtained from the x-intercepts of the plot.

concentrations were satisfactorily fit to a single exponential process.

The kinetic effects of anion binding can be explained by the formation an inactive complex (EI) upon anion (I) binding (eq 2)[16] that is incapable of undergoing intramolecular ET (eq 3). Free photoreduced enzyme (E) is generated by ligand dissociation from this complex (eq 2). If $k_{obsd} = k_{ET}[E]$, $K_i = [E][I]/[EI]$,

$$EI \text{ (inactive)} \overset{K_i}{\rightleftharpoons} E + I \quad (2)$$

$$E \overset{k_{ET}}{\rightleftharpoons} P \quad (3)$$

and k_{ET} is defined as the rate constant for intramolecular ET in the absence of anions,[17] then substitution for [E] using the expression for K_i gives

$$\frac{1}{k_{obsd}} = \frac{[I]}{K_i k_{ET}} + \frac{1}{k_{ET}} \quad (4)$$

Thus, a plot of $1/k_{obsd}$ vs [I] will give a straight line with a slope of $1/K_i k_{ET}$ and an intercept of $1/k_{ET}$, as shown in Figure 2 for SO_4^{2-} and Cl^-.

These results verify that an anion binding site exists on sulfite oxidase and provide direct kinetic evidence that intramolecular ET is inhibited by anion binding. The structural basis for this effect is currently unknown because there is as yet no X-ray structural data available for sulfite oxidase (or any other pterin-containing molybdenum enzyme).[18] The inhibition of sulfite oxidase turnover by anions under steady-state conditions may also be due to limitations on intramolecular ET because similar anion binding constants are obtained for both the steady-state[10] and flash photolysis experiments. Previous evidence for similar kinetic effects in redox enzymes or metalloproteins comes from a lack of dependence of rates of ET on driving force,[4c] the facilitation of ET upon the binding of a ligand,[4e,f] and surface diffusion within electrostatic complexes.[4g]

Acknowledgment. This work was supported by NIH Grants DK15057 (to G.T.) and GM37773 (to J.H.E.) and National Research Service Award GM14689-01 (to E.P.S.).

(16) The ET rate of the inactive species need not be zero, but it must be smaller than the slowest rate observed at high salt concentration because all of the ET reactions can be satisfactorily fit by a single exponential.

(17) In these equations, E represents the Fe(II),Mo(VI) species created upon flash photolysis, and P the Fe(III),Mo(V) species resulting from intramolecular electron transfer. k_{ET} represents the sum of the forward and reverse rate constants for this process.

(18) See ref 3c for a discussion of the effect of distance on ET rates.

(19) Tris: tris(hydroxymethyl)aminomethane, $pK_a = 8.1$; Bis-Tris: [bis-(2-hydroxyethyl)amino]tris(hydroxymethyl)methane, $pK_a = 6.5$; Bis-tris propane: 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane, $pK_{a_1} = 6.8$, $pK_{a_2} = 9.0$.

The Intrinsic Competition between Elimination and Substitution Mechanisms Is Controlled by Nucleophile Structure

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Received July 31, 1992

Whereas the competition between S_N2 and E2 mechanisms is among the best understood reactivity–selectivity relationships in the liquid phase,[1] the gas-phase anion–molecule counterpart[2-10] is much less well defined. Gas-phase studies are hindered because the readily measured ionic product is identical for both mechanisms while the distinct neutral products are extremely difficult to detect. The difficulty of precisely controlling the interaction energy further complicates some gas-phase investigations. Recent FA-SIFT studies using localized heteroatomic bases with simple alkyl halides concluded that elimination is often kinetically favored over substitution.[11,12] Substitution, however, is preferred for sulfur anions reacting with alkyl halides, and substitution effectively competes with elimination when the localized base H_2N^- reacts with ethyl methyl sulfite.[13] We wish to report that the competition between elimination and substitution at simple alkyl centers depends, most importantly, on the type of base used rather than its thermodynamic basicity; localized heteroatomic bases prefer to react with ethyl dimethyl phosphate by elimination while delocalized carbon bases prefer substitution.

Ethyl dimethyl phosphate[14] is an ideal substrate for these competition studies. First, it contains a common, excellent leaving group,[15] $\Delta H°_{acid}[(RO)_2POOH] \approx 332$ kcal mol^{-1}, for both substitution and elimination channels. Second, the competition of interest is "intramolecular", obviating the need to consider differences in ion–neutral dynamics or energetics when distinct substrates are used. Third, reaction at phosphorus is negligible[15] while substitution and elimination reactions are facile. Fourth,

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