Electron Transfer in Sulfite Oxidase: Effects of pH and Anions on Transient Kinetics†

Eric P. Sullivan, Jr.,‡ James T. Hazzard,§ Gordon Tollin,*,§ and John H. Enemark*,‡

Departments of Chemistry and Biochemistry, The University of Arizona, Tucson, Arizona 85721

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ABSTRACT: Intramolecular electron transfer (ET) rates in sulfite oxidase (SO) were measured using flavin semiquinone reductants [5-deazariboflavin (dRFH•) and lumiflavin (LFH•)] generated by laser flash photolysis. Rapid bimolecular reduction of the heme by the dRF semiquinone occurred ($k = 4 \times 10^8$ M^{-1} s^{-1} at pH 6; 1×10^8 M^{-1} s^{-1} at pH 9), followed by heme Fe(II) reoxidation due to intramolecular electron transfer to Mo(VI). Flash-induced difference spectra indicated that only spectral processes due to reduction and oxidation of the *b*-type heme prosthetic group were observed, with no detectable spectral contribution from the Mo cofactor. The extent of reoxidation decreased greatly from pH 6 to 9 (50% to 3%), as expected from the shifts in the redox potentials of the heme and Mo cofactor with pH, consistent with an electron transfer equilibrium between the two redox centers. The observed rate constant for the Fe(II) to Mo(VI) electron transfer decreased from 1650 s^{-1} at pH 6 to 60 s^{-1} at pH 9 and showed a maximum of 2400 s^{-1} at pH 7. Increases in salt concentration greatly decreased intramolecular ET rate constants (direct reduction by flavin semiquinone was unchanged), due to the binding of anions. Titration with the sodium salts of Cl^-, SO_4^{2-}, and $H_2PO_4^-/HPO_4^{2-}$ resulted in decreases in rate constants of intramolecular ET from 1500 s^{-1} to <100 s^{-1} at pH 6 and 7. Similar dissociation constants were measured for the binding of these anions by flash photolysis and by steady-state enzyme kinetics using the inhibition of the sulfite/cytochrome *c* assay reaction for sulfite oxidase. A mechanism is proposed in which anion binding to the enzyme inhibits the rate of intramolecular electron transfer.

Sulfite oxidase (SO)[1] catalyzes the oxidation of sulfite to sulfate with the reduction of two equivalents of ferricytochrome *c* (eq 1), the terminal step in the metabolism of sulfur-containing amino acids (Howell & Fridovich, 1968; Cohen & Fridovich, 1971; Rajagopalan, 1980):

$$SO_3^{2-} + H_2O + 2\text{Fe(III) cyt } c \rightleftarrows SO_4^{2-} + 2\text{Fe(II) cyt } c + 2H^+ \quad (1)$$

Ferricyanide and dioxygen can also serve as terminal electron acceptors. The enzyme contains a pterin–molybdenum cofactor (Rajagopalan, 1991; Rajagopalan & Johnson, 1992) at the catalytic site and a *b*-type heme in a separate domain which is similar in sequence to cytochrome b_5 (Neame & Barber, 1989). Evidence exists that as the enzyme turns over, electrons are shuttled from sulfite to the molybdenum center to the heme and then to cytochrome *c* (Speck et al., 1981). A proposed scheme for sulfite oxidase catalysis is shown in Figure 1 (Rajagopalan, 1980).

Anions such as SO_4^{2-}, Cl^-, and HPO_4^-/HPO_4^{2-} have been shown to be inhibitors of the flow of electrons from sulfite to cytochrome *c* and ferricyanide but not to O_2 (Kessler & Rajagopalan, 1974). This suggests that the action of these anions may be to inhibit ET from the Mo cofactor to the heme and that O_2 reacts directly at the Mo cofactor and is thus unaffected by the rate of internal ET. These findings caused Cohen and Fridovich (1971) to propose (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 one-electron acceptors".

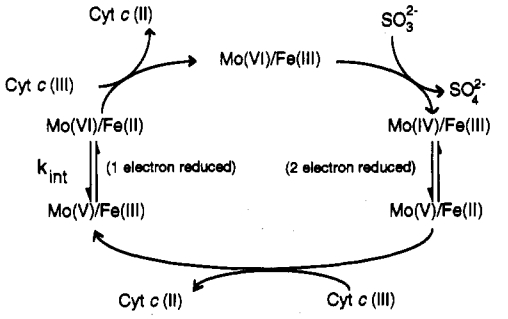

FIGURE 1: Proposed mechanism for the oxidation of sulfite by sulfite oxidase [adapted from Rajagopalan (1980)].

A series of studies using EPR spectroscopy has demonstrated that anions bind to the Mo(V) form of SO (Cramer et al., 1979; George, 1985; Bray et al., 1982, 1983; Lamy et al., 1980; Gutteridge et al., 1980). Low pH and high chloride concentrations generate the "low-pH" form of the enzyme, while high pH and low anion concentration gives rise to the "high-pH" form. In the presence of phosphate a third species is observed (George et al., 1988). Thus, the environment of the molybdenum atom is affected by both pH and the presence and nature of anions.

X-ray absorption and EXAFS spectra have been used to probe the structure of SO in the Mo(IV), (V), and (VI) oxidation states in buffer of pH 9/low chloride and pH 6/high

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‡ Department of Chemistry.

§ Department of Biochemistry.

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[1] Abbreviations: ET, electron transfer; SO, sulfite oxidase; dRF, 5-deazariboflavin; LF, lumiflavin; dRFH•, deazariboflavin semiquinone; LFH•, lumiflavin semiquinone; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

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chloride (0.3 M) (George et al., 1989, 1990). The effects of changing the buffer were largest for the Mo(IV) and Mo(V) states and smallest for the Mo(VI) state. This indicates that anions bind more strongly to the Mo(V) and Mo(IV) oxidation states. The study concluded that in the Mo(VI) oxidation state the molybdenum atom possesses two terminal oxo groups (Mo=O) and approximately three thiolate ligands, while the Mo(V) and Mo(IV) states each have one oxo and one hydroxyl (Mo–OH) ligand.

Microcoulometric titrations of SO at several pH and salt compositions have shown that the potential of the Mo(VI/V) couple is more positive than that of the Fe(III/II) at low pH but becomes less positive at high pH (Spence et al., 1991). Binding of chloride to the Mo(V) state should also shift the potential for the Mo(VI/V) couple to a more positive value. Therefore, upon reduction of Fe(III) to Fe(II) using flash photolysis, subsequent electron transfer to Mo(VI) should be favored at pH 6 but not at pH 9.

SO presents a unique system to study intramolecular ET. The redox potential of one of the sites can be altered by changing the pH, and the well-documented inhibition of steady-state turnover by anions has been attributed to interference with ET (Cohen & Fridovich, 1971; Kessler & Rajagopalan, 1974). By use of the technique of laser flash photolysis with flavin radical reductants (Tollin & Hazzard, 1991), intramolecular ET can be observed in the absence of substrate and external electron acceptors. Preliminary studies using this methodology with SO have previously been reported (Kipke et al., 1988; Sullivan et al., 1992). In the present experiments we have explored the dependence of the rate constant and extent of intramolecular ET on pH and salt concentrations.

EXPERIMENTAL PROCEDURES

Sulfite oxidase was purified by the method of Kipke et al. (1989) with several modifications. Raw chicken liver obtained fresh from a local wholesaler was substituted for liver acetone powder. Livers (2.3 kg) were homogenized in a Waring blender in approximately 5 volumes of 25 mM phosphate buffer and 0.1 mM PMSF, pH 8.0 and centrifuged. Ammonium sulfate precipitations and DE-52 and phenyl-Sepharose chromatography steps were performed as described previously. The final G-200 gel permeation step was replaced by affinity chromatography on a cytochrome *c*-Sepharose column (1 × 25 cm) (Ritzmann & Bosshard, 1988). A gradient of 5–50 mM phosphate buffer, pH 8, was used to elute the enzyme. This improved preparation typically yielded 35 mg of purified sulfite oxidase, with a heme to protein absorbance ratio (A_{414}/A_{280}) of >0.8 and specific activity of 75 units/mg (determined using the sulfite/cytochrome *c* assay reaction). Further purification could be achieved using a Mono Q FPLC column (Pharmacia) with a gradient of 0–100 mM NaCl in bis-Tris buffer at pH 6.4; fractions with $A_{414}/A_{280} = 1$ were isolated. Enzyme solutions were concentrated using a Centricon 30 (Amicon) ultrafiltration device and stored as concentrated (1 mM) solutions frozen in liquid nitrogen until use. The enzyme subunits migrated as a single band of approximately 50–55 kDa in SDS–polyacrylamide gels. Similar levels of purification have recently been reported by Bellissimo and Rajagopalan (1991).

A matrix-assisted laser desorption time of flight mass spectrum (Chait & Kent, 1992) was taken of a highly purified enzyme sample. Spectra were obtained on a Finnegan LASERMAT spectrometer calibrated externally using α-lactalbumin and using a matrix of sinapinic acid. We attribute a large peak at 51 529 Da to the intact SO subunit. This is consistent with the mass calculated for the published amino acid sequence of SO (Neame & Barber, 1989) plus that of the heme and Mo cofactor. A much smaller peak at 103 kDa is assigned to the intact SO dimer. These values are smaller than those previously obtained using SDS–PAGE and gel-permeation studies (Cohen & Fridovich, 1971).

Steady-state enzyme kinetics were performed using the sulfite/cytochrome *c* assay reaction for sulfite oxidase and monitoring the reduction of cytochrome *c* at 550 nm. Concentrations of inhibitor and sulfite were varied and plots of 1/rate vs 1/substrate were made for a series of inhibitor concentrations. K_i was determined for each inhibitor from the *x* intercept of a replot of the slopes of the lines vs inhibitor concentration. (Plots are available as supplementary material.)

Laser flash photolysis experiments were performed anaerobically using 0.25 or 0.5 mL of buffer solution containing approximately 90 μM 5-deazariboflavin (dRF) or lumiflavin (LF) and EDTA (2.5–10 mM). Kinetic traces were generally obtained at 555 nm, corresponding to the λ_{max} for the reduced form of the heme. Enzyme concentrations were determined by using an extinction coefficient of 99 900 M^{-1} cm^{-1} at 413 nm per subunit for SO (Cohen & Fridovich, 1971). Concentration dependence studies and salt titrations were performed by injecting a concentrated protein or salt stock solution via Hamilton syringe and deoxygenating the cuvette before recording kinetic data. pH dependence studies were conducted in a universal buffer of 6 mM Tris, 6 mM bis-Tris, 6 mM bis-Tris propane and 5 mM EDTA adjusted with acetic acid or in 5 mM bis-Tris and 5 mM EDTA adjusted with acetic or *p*-toluenesulfonic acid. A description of the laser flash apparatus has been published previously (Tollin et al., 1986; Hazzard et al., 1991). Transient absorbance changes were analyzed using the computer fitting procedure SIFIT, obtained from OLIS Inc., Jefferson, GA. The flash-induced difference spectrum was recorded by measuring the absorbance change from the preflash baseline at 0 (extrapolated value) and 10 ms after the laser pulse for each wavelength.

The flash photolysis experiment involves the rapid (<1 μs) generation of a strongly reducing flavin or deazaflavin semiquinone. The amount of semiquinone is small compared to enzyme concentration and therefore pseudo-first-order conditions exist for the reaction of flavin semiquinone and enzyme, and a given protein molecule receives no more than a single electron during the laser flash (Tollin & Hazzard, 1991).

RESULTS AND DISCUSSION

The following scheme (eqs 2–4) describes the kinetics observed in the flash photolysis experiments. Deazariboflavin (dRF) or lumiflavin (LF) semiquinone (dRFH•, LFH•) is generated by the laser pulse in the presence of sacrificial donor (EDTA) (eq 2). The strongly reducing flavin semiquinone

$$\mathrm{dRF} \xrightarrow[\mathrm{EDTA}]{h\nu} \mathrm{dRFH^{\bullet}} \qquad (2)$$

$$\mathrm{dRFH^{\bullet} + SO(Fe^{III},Mo^{VI}) \xrightarrow{k_{12}} dRF + SO(Fe^{II},Mo^{VI})} \qquad (3)$$

$$\mathrm{SO(Fe^{II},Mo^{VI}) \overset{k_{int}}{\rightleftharpoons} SO(Fe^{III},Mo^{V})} \qquad (4)$$

(E_m = –650 mV for dRFH•/dRF) rapidly reduces the heme center of sulfite oxidase generating the one-electron-reduced form of the enzyme (eq 3). The rate constant for this process

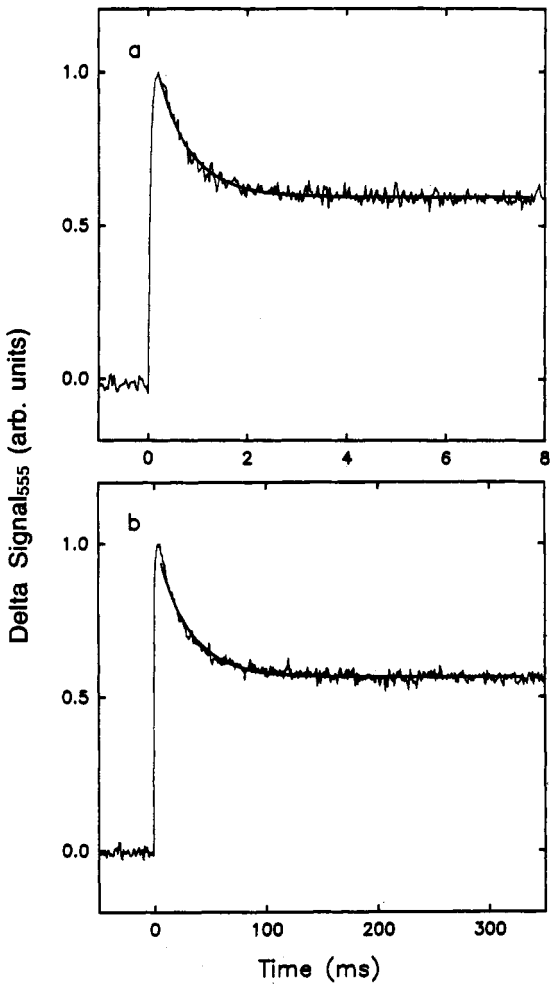

FIGURE 2: Kinetic traces obtained at pH 6 (555 nm) illustrating heme photoreduction and reoxidation upon laser flash photolysis of sulfite oxidase (50 μM), using 5-deazariboflavin (90 μM) as a reductant. (a) 0 added sulfate, 15-ms time scale; k_{obs} for heme reoxidation is 1580 s^{-1}. (b) 23 mM sulfate, 500-ms time scale; k_{obs} = 35 s^{-1}. Addition of salt greatly decreases the rate of intramolecular ET due to the binding of anions. The solid line indicates a single-exponential fit to the reoxidation kinetics.

is designated k_{12}. Equilibration can then occur between the two redox centers of the enzyme according to their relative redox potentials (eq 4). The rate constant for attaining this equilibrium is designated k_{int}, which represents the sum of the forward and reverse rate constants.

Effect of pH on Electron Transfer. Figure 2a shows a typical kinetic transient observed upon excitation of a solution containing dRF and oxidized SO in the presence of EDTA at pH 6.0. The rapid increase in absorbance at 555 nm corresponds to direct reduction of the heme prosthetic group of the enzyme by dRFH$^{\bullet}$. The observed rate constant for this reaction is linearly dependent on [SO] and the calculated second-order rate constant (listed in Table I) has a value of 4×10^8 M^{-1} s^{-1}. A decrease in absorbance on a longer time scale is also observed. This second kinetic phase is enzyme concentration independent, and thus is taken to represent intramolecular electron transfer from heme Fe(II) to Mo(VI) as given in eq 4. The rate constant (k_{int}) and extent of heme reoxidation are 1650 s^{-1} and 50%, respectively.

Flash-induced difference spectra obtained 0 and 10 ms after laser excitation are shown in Figure 3. Within the resolution of the experiment, peak wavelengths and isosbestic points are identical to the steady-state difference spectrum for heme reduction (Kipke et al., 1988). Furthermore, although the

Table I: Rate Constants of Electron Transfer for Sulfite Oxidase

pH	k_{12} (dRFH$^{\bullet}$) (M^{-1} s^{-1})	k_{12} (LFH$^{\bullet}$) (M^{-1} s^{-1})	k_{int} (s^{-1})
6	$(4 \pm 1) \times 10^8$ [a]	$(3.3 \pm 0.2) \times 10^7$ [a]	$(1.65 \pm 0.04) \times 10^3$ [b]
7	$(2 \pm 1) \times 10^8$ [c]		$(2.4 \pm 0.2) \times 10^3$ [b]
8			$(8.3 \pm 0.5) \times 10^2$ [d]
9	$(1.1 \pm 0.5) \times 10^8$ [a]	$(7.8 \pm 0.8) \times 10^7$ [a]	$(6 \pm 3) \times 10^1$ [a]

[a] 18 mM bis-Tris and 5 mM EDTA. [b] 5 mM universal buffer and 2.5 mM EDTA. [c] 20 mM phosphate buffer and 10 mM EDTA. [d] 5 mM Tris and 0.5 mM EDTA.

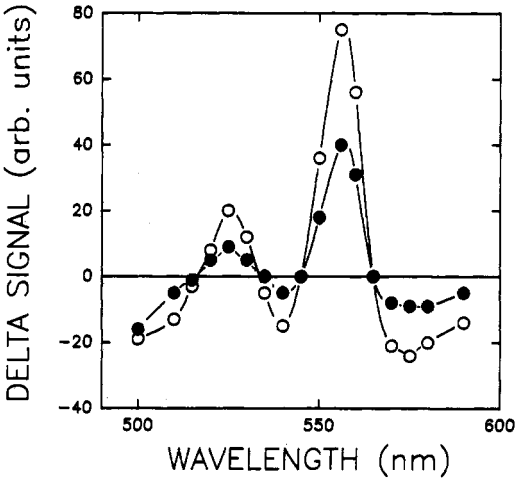

FIGURE 3: Flash-induced difference spectrum of sulfite oxidase (reduced-oxidized) obtained at 0 (extrapolated) (○) and 10 (●) ms after the laser flash. Buffer conditions were 5 mM bis-Tris, pH 7.0, 0.5 mM EDTA, and 90 μM 5-deazariboflavin (dRF). Within the resolution of the experiment, the flash-induced difference spectrum is identical to that obtained using steady-state methods. Difference spectra obtained at longer time scales were similar in appearance.

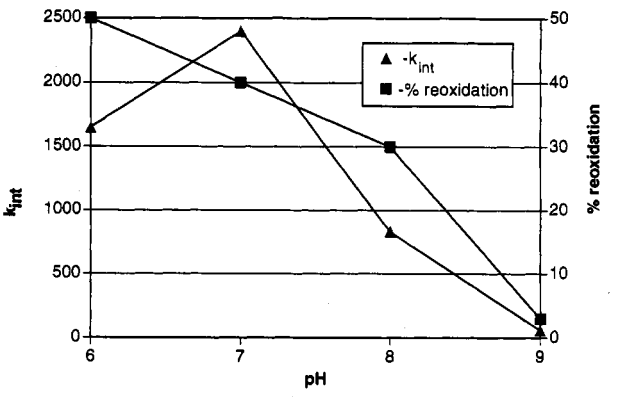

FIGURE 4: Effects of pH on k_{int} (▲) and % reoxidation (■) in sulfite oxidase.

magnitude of the slow kinetic phase has a dependence on pH (see below), the overall spectral characteristics of the transients were not affected by either pH or buffer conditions. No spectral changes directly attributable to the Mo cofactor were observed, presumably due to its much lower absorptivity (Johnson & Rajagopalan, 1977). Thus, the spectra confirm that the transient changes observed in these experiments are directly related to heme redox chemistry.

At pH 7, a 50% decrease in the rate constant for direct enzyme reduction by dRFH$^{\bullet}$ was obtained compared to the data at pH 6 (Table I). Furthermore, there was a marked increase in k_{int} to a value of 2400 s^{-1}, although the extent of reoxidation decreased to 40%. Figure 4 shows plots of k_{int} and extent of heme oxidation vs pH. As the pH was increased from 7 to 8, the value of k_{int} decreased to 830 s^{-1}, concomitant

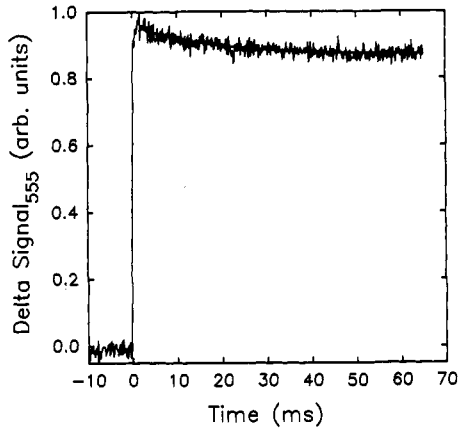

FIGURE 5: Transient kinetics at pH 9 (555 nm), 18 mM universal buffer, 90 μM dRF, 55 μM sulfite oxidase, 75-ms time scale. k_{obs} for the reoxidation phase is 60 s^{-1} and accounts for 3% of the total signal. The small amount and slow rate of reoxidation of the heme iron is consistent with a significantly more negative potential for the Mo(VI/V) couple relative to the heme at this pH.

with a decrease in the extent of oxidation to ~30%. At pH 9 (Figure 5), k_{int} = 60 s^{-1} and only 3% oxidation was observed. At pH 9, the rate constant for direct reduction of the enzyme by dRFH$^{\bullet}$ (k_{12}) was also further decreased (Table I).

The above results demonstrate that all three parameters measured in these experiments (k_{12}, k_{int}, and % reoxidation) show a significant pH dependence. The second-order rate constant for direct reduction of SO by dRFH$^{\bullet}$ decreased with increasing pH, consistent with the observed decrease in midpoint potential of the heme Fe in going from pH 9 (E_m = 90 mV) to pH 6 (E_m = 39 mV) (Spence et al., 1991). The results may also indicate a change in the heme accessibility as the pH is decreased; however, we have not performed experiments to directly address this question. It is also interesting to note that no evidence was obtained for direct reduction of the Mo cofactor by free dRFH$^{\bullet}$ in these experiments. Similarly, electrogenerated mediator oxidants such as $[Co(Me_xphen)_3]^{3+}$ (Coury et al., 1991; Yang et al., 1993) have also been shown to react solely at the heme center, and cytochrome *c*, the physiological electron acceptor, interacts with the *b*-type cytochrome domain. Taken together, the data suggest a higher degree of accessibility of the cytochrome heme, relative to the Mo cofactor, to large redox agents; however, the Mo cofactor has been shown to interact with small anionic species such as sulfite, sulfate, phosphate, chloride, and ferricyanide (Kessler & Rajagopalan, 1974; Gardlik & Rajagopalan, 1991).

The observed decrease in the percentage of heme reoxidation by the Mo cofactor is also consistent with the decrease in midpoint potential of the Mo cofactor with increasing pH. However, at pH 6, there is a significant difference between the experimentally observed percent reoxidation for equation 4 (50%) and that calculated from the 41 mV difference between the approximate midpoint potentials for the heme and Mo cofactors (Spence et al., 1991). This potential difference should yield a ratio of Fe(III),Mo(V)/Fe(II),Mo(VI) of 4.6, which corresponds to eq 4 proceeding 83% to completion. Our data suggest that the two centers are approximately isopotential (assuming that equilibrium is established over a time range of approximately 1 s in the flash experiment). The reason for this discrepancy between the observed and predicted positions of equilibria may involve several factors. The reported midpoint potentials (Spence et al., 1991) were measured at high ionic strength and represent successive reductions of the enzyme using dye mediators. These potentials can only be used as a guide for interpreting the one-electron flash photolysis experiments because the microcoulometry potentials may include cooperative effects on the potential of one redox site due to the prior reduction of the other site. Such cooperativity cannot occur in the one-electron flash photolysis experiment. Furthermore, it is not experimentally possible to measure the potentials using microcoulometry at the low anion concentrations under which the flash photolysis experiments were performed. Preferential anion binding to Mo(V) relative to Mo(VI) should shift the potential to a higher value. The expression for the dependence of the Mo(VI/V) potential upon pH and Cl^- concentration suggests that the Mo(VI/V) and Fe(III/II) couples will be within ~10 mV under the conditions shown in Figure 2 (Spence et al., 1991).

The data of Figure 4 also clearly show that there is an optimum pH for intramolecular electron transfer within SO; k_{int} at pH 7 (2400 s^{-1}) is significantly faster than at pH 6 (1650 s^{-1}). This apparent pH dependence of k_{int} is difficult to separate from the effects of anion binding, and the observed maximum in k_{int} may be influenced by a higher anion concentration present in the 5 mM bis-Tris buffer at pH 6 than at pH 7 since strong acids are used to adjust the pH. (Even weakly coordinating anions such as perchlorate and *p*-toluenesulfonate were shown to inhibit intramolecular ET or steady-state kinetics.) The effects of anions on intramolecular ET rate constants are discussed below.

Titration with Anions. Addition of salts generally decreased the rate of the reoxidation phase, whereas the rate constant for the fast reduction by flavin semiquinone was unchanged. For example, addition of sodium sulfate to 23 mM decreased the rate of the reoxidation from 1580 (Figure 2a) to 35 s^{-1} (Figure 2b) at pH 6. Titrations with the sodium salts of various anions (Cl^-, SO_4^{2-}, and $H_2PO_4^-/HPO_4^{2-}$) were performed by addition of a concentrated stock solution of salt to the flavin/enzyme solutions and recording the transient kinetics after each addition. For Cl^- and SO_4^{2-}, the kinetics for the reoxidation were well behaved and were satisfactorily fitted with a single exponential for all anion concentrations. Additions of a concentrated phosphate buffer showed monophasic kinetics for concentrations below 1.87 mM and biphasic above this concentration, possibly due to the ability of $H_2PO_4^-$ and HPO_4^{2-} to bind with different affinities. The extent of heme reoxidation remained constant at all salt concentrations, indicating that anion binding has a kinetic rather than a thermodynamic influence and inhibits both the forward and reverse microscopic rate constants for equilibrium to the same extent.

The kinetic effects of anion binding can be explained by the following scheme, in which binding of anions (I) results in an inactive complex (eq 5), incapable of undergoing intramolecular ET (eq 6). Free enzyme (E) is generated by ligand dissociation from this complex and the observed rate constant will be proportional to the free enzyme concentration.[2]

$$\mathrm{EI\ (inactive)} \overset{K_i}{\rightleftharpoons} \mathrm{E} + \mathrm{I} \qquad (5)$$

$$\mathrm{E} \overset{k_{ET}}{\rightleftharpoons} \mathrm{P} \qquad (6)$$

If $k_{obs} = k_{ET}[E]$, $K_i = [E][I]/[EI]$, and k_{ET} is defined as the

[2] In these equations E represents the Fe(II), Mo(VI) species created upon flash photolysis, and P represents 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.

Table II: Dissociation Constants for Anions from Sulfite Oxidase

anion	flash (mM)	steady state (mM)
Cl^- [a]	4 ± 1	8 ± 3
SO_4^{2-} [a]	0.8 ± 0.2	0.8 ± 0.3
$H_2PO_4^-/HPO_4^{2-}$ [b]	1.2 ± 0.2	0.7 ± 0.4

[a] 18 mM universal buffer, pH 6. [b] 18 mM universal buffer, pH 7.0.

rate constant in the absence of inhibitor, then substitution for [E] using the expression for K_i gives the following rate equation:

$$\frac{1}{k_{obs}} = \frac{[I]}{K_i k_{ET}} + \frac{1}{k_{ET}} \quad (7)$$

Thus, a plot of $1/k_{obs}$ vs [I] will give a straight line with a slope of $1/K_i k_{ET}$. Linear plots of this type were obtained for Cl^-, SO_4^{2-}, and $H_2PO_4^-/HPO_4^{2-}$ (plots are presented in the supplementary material) and the binding constants are reported in Table II. Dianions are shown to bind more strongly than monoanions. This is consistent with the results of the steady-state enzyme kinetic experiments (this work; Kessler & Rajagopalan, 1974). At low anion concentrations, linear plots of $1/k_{obs}$ vs anion concentration were obtained, while at higher concentrations (>50 mM), the effects of anion binding level off. This may indicate that the anion-bound species is not completely inactive to intramolecular ET but that it occurs at a greatly decreased rate. There is little data available on the rates of anion exchange of Mo cofactor-containing enzymes. Studies of ligand exchange of model oxo-molybdenum complexes suggest that the rates increase with oxidation state and range from 10^0 for Mo(IV) to 10^6 M^{-1} s^{-1} for Mo(VI) complexes (Saito & Sasaki, 1982). There is no evidence available to suggest whether anion exchange is rapid or slow relative to electron transfer in sulfite oxidase, and this may be an interesting topic for future studies.

Another possible mechanism for the inhibition of electron transfer rates is that anion binding influences the potential of the Mo cofactor by shifting the equilibrium in eq 4. EPR, EXAFS, and coulometric studies of intact SO suggest that anions bind more strongly to the Mo(V) state than to Mo(VI). On the other hand, Johnson and Rajagopalan (1977) observed that the visible spectrum of the oxidized molybdenum domain [Mo(VI)] produced by tryptic cleavage of SO was dependent upon pH and anions. For our flash photolysis experiments on the millisecond time scale it is not known whether anion binding preferentially stabilizes Mo(V) or Mo(VI) and whether the Mo(VI/V) redox potential changes relative to that of the heme. However, the lack of dependence of the observed equilibrium (Figure 2) on anion concentration is *inconsistent* with a mechanism in which the inhibition of ET rates is caused by a shift in the potential of the Mo cofactor. Yang et al. (1993) also observed decreases in the rate of intramolecular electron transfer with addition of anions. Their experiments using electrogenerated mediator oxidants and SO in the presence of excess sulfite probably measured ET in both the one- and two-electron-reduced forms of the enzyme.

Steady-State Kinetics. Inhibition constants were also measured under steady-state conditions for Cl^-, SO_4^{2-}, and $H_2PO_4^-/HPO_4^{2-}$ using the sulfite/cytochrome *c* assay reaction in similar buffer to that used in the flash photolysis experiments. All inhibitors showed mixed-type inhibition (Segel, 1976) vs sulfite, and K_i values were smaller than those measured by Kessler and Rajagopalan (1974) in higher ionic strength buffer. The dissociation constants measured using flash photolysis and steady-state enzyme kinetics are compared in Table II. The K_i values determined using flash photolysis titration and steady-state kinetics generally agree within experimental error. This suggests that anion binding to the same site is being observed using each method and that the mechanism of inhibition is to diminish the rate of intramolecular ET during enzyme turnover. The redox potentials and electron transfer studies are consistent with the observation that steady-state turnover occurs faster at higher pH and is optimal at pH 8 (for chicken liver SO; Speck et al., 1981) and 8.6 (for beef liver SO; Cohen & Fridovich, 1971), because at higher pH the equilibrium of eq 4 is strongly shifted to the left, which is in the forward direction with regard to enzyme turnover.

A previous study of electron transfer in SO found that reduction of the heme group occurred using lumiflavin semiquinone ($LFH^\bullet$), followed by intramolecular ET from Fe(II) to Mo(VI) (Kipke et al., 1988). Using $dRFH^\bullet$, the second kinetic phase was additional heme reduction, attributed to ET from Mo(V) to Fe(III) (this process could occur if flavin reacted at both the Mo cofactor and the heme). In the present study, we have observed that the second kinetic phase using $dRFH^\bullet$ is intramolecular ET from Fe(II) to Mo(VI), similar to that observed previously using $LFH^\bullet$. The result reported by Kipke et al. (1988) was apparently an experimental artifact, possibly due to an impurity in the flavin solution or in the enzyme preparation that caused a slower additional reduction phase. It should be noted that the authors did not realize the effects on the rate of ET by anion binding at the time and that the reported rate for Fe(II) to Mo(VI) of 155 s^{-1} (using the $LFH^\bullet$ reductant) shows the effect of the 20 mM phosphate used in the experiment.

The possibility that structural rearrangements occurring within proteins or electrostatic protein–protein complexes can limit the observed rate of electron transfer has been reviewed (Brunschwig & Sutin, 1989; Hoffman & Ratner, 1987). The mixed-type inhibition of enzyme turnover observed for anions indicates that they do not directly compete with substrate. The results from EPR spectroscopy show that anion binding occurs at the Mo cofactor. This evidence supports the following conclusions: (1) an anion binding site exists at the Mo cofactor but may be separate from the active site and (2) anion binding to this site results in a greatly reduced rate of intramolecular ET. It is possible that the ET rate observed in SO in the absence of added anions is the true ET rate, while upon the addition of anions the rate is governed by the rate of some other process such as anion dissociation to produce the active form. A possible explanation is that anion binding may induce a conformational change that creates an inactive complex. However, no evidence currently exists as to the type of physical change that occurs upon the binding of anions to reduce the rate of intramolecular ET. Previous evidence for similar effects in redox enzymes or proteins comes from a lack of dependence of rates of ET on driving force (McLendon et al., 1987), rates of diffusion of small molecules through hemoglobin (Feitelson & McLendon, 1991), the facilitation of ET upon the binding of a ligand (Walker & Tollin, 1991; Walker & Tollin, 1992), and surface diffusion within electrostatic complexes (Zhou & Kostic, 1992).

Sulfite oxidase has several features in common with flavocytochrome b_2 (L-lactate dehydrogenase) (Chapman et al., 1991), the crystal structure of which has been determined (Xia & Mathews, 1990). Both molecules contain a *b*-type heme and one other cofactor at the active site (FMN for flavocytochrome b_2) and catalyze two-electron oxidations, transferring electrons from the active site to the heme and then to cytochrome *c*. The structure for flavocytochrome b_2 reveals that the heme and the flavin are coplanar and are

separated by 9.7 Å. Walker and Tollin (1991) found that ET from the heme to the FMN occurred in the one-electron-reduced enzyme only in the presence of pyruvate. The X-ray structure shows that enzyme subunits with pyruvate bound at the active site have a disordered cytochrome b_5 domain, suggesting a high degree of mobility of this region. In sulfite oxidase, anion binding (presumably at the Mo center) slows intramolecular ET. It is interesting that the measured rate constants for intramolecular ET for SO (in the absence of added anions) and flavocytochrome b_2 in the presence of pyruvate (Walker & Tollin, 1992) are similar, and this may suggest that ET occurs over a similar distance in these two enzymes (Moser et al., 1992).

Sulfite oxidase presents an interesting and complex system in which to study biological ET reactions. The redox equilibrium can be controlled by changing the pH, while the binding of anions serves to limit the rate of intramolecular ET. The ability to titrate the rate of electron transfer confirms that an anion binding site exists on sulfite oxidase and shows that intramolecular ET is inhibited by anion binding. Because anions also inhibit enzyme turnover and similar binding constants were observed using steady-state enzyme kinetics, it appears that their effect is to limit ET during enzyme turnover.

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SUPPLEMENTARY MATERIAL AVAILABLE

Four figures showing plots of $1/k_{obs}$ vs anion concentration for sulfate, chloride, and phosphate and plots of 1/rate vs 1/substrate and replots of the slopes of the lines vs inhibitor concentration used for the determination of K_i values (5 pages). Ordering information is given on any current masthead page.

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