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Chicken Liver Sulfite Oxidase. Kinetics of Reduction by Laser-Photoreduced Flavins and Intramolecular Electron Transfer[†]

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Received August 21, 1987; Revised Manuscript Received December 2, 1987

ABSTRACT: Laser flash photolysis was used to study the reaction of photoproduced 5-deazariboflavin (dRFH·), lumiflavin (LFH·), and riboflavin (RFH·) semiquinone radicals with the redox centers of purified chicken liver sulfite oxidase. Kinetic studies of the native enzyme with dRFH· yielded a second-order rate constant of 4.0×10^8 M^{-1} s^{-1} for direct reduction of the heme and a first-order rate constant of 310 s^{-1} for intramolecular electron transfer from the Mo center to the heme. The reaction with LFH· gave a second-order rate constant of 2.9×10^7 M^{-1} s^{-1} for heme reduction. Reoxidation of the reduced heme due to intramolecular electron transfer to the Mo center gave a first-order rate constant of 155 s^{-1}. The direction of intramolecular electron transfer using dRFH· and LFH· was independent of the buffer used for the experiment. The different first-order rate constants observed for intramolecular electron transfer using dRFH· and LFH· are proposed to result from chemical differences at the Mo site. Flash photolysis studies with cyanide-inactivated sulfite oxidase using dRFH· and LFH· resulted in second-order reduction of the heme center with rate constants identical with those obtained with the native enzyme, whereas the first-order intramolecular electron-transfer processes seen with the native enzyme were absent. The isolated heme peptide of sulfite oxidase gave only second-order kinetics upon laser photolysis and confirmed that the first-order processes observed with the native enzyme involve the Mo site. The flash-induced difference spectrum of native sulfite oxidase using dRFH· and LFH· resulted in absorbance increases in the 530–570-nm region of the spectrum that were not present in the static difference spectrum of the enzyme. These absorbances are proposed to be associated with the Mo center.

Sulfite oxidase is a dimeric molybdenum-containing protein that catalyzes the oxidation of sulfite to sulfate (Rajagopalan, 1980). The enzyme contains two molybdenum–pterin centers and two cytochrome b_5 type hemes per molecule (Cohen et al., 1971; Cohen & Fridovich, 1971b; Johnson et al., 1977), and catalysis occurs at the molybdenum site (Johnson et al., 1974a,b). Although sulfite oxidase has considerably fewer redox sites than the kinetically well-studied molybdenum-containing protein xanthine oxidase [cf. Hille and Massey (1986)], the transient kinetics of the redox chemistry of the enzyme have yet to be reported.

The proposed catalytic cycle of sulfite oxidase requires the fully reduced Mo site to undergo two, one-electron, intramo-

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[†] This work was supported in part by grants from the National Institutes of Health [GM 21277 (to M.A.C.), AM 15057 (to G.T.), and GM 37773 (to J.H.E.)] and by the University of Arizona Agricultural Experiment Station [AZT 174510 (to R.A.S.)].

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lecular electron-transfer processes between the molybdenum and the b_5-type heme in order to regenerate a catalytically active form of the enzyme [cf Rajagopalan (1980)]. Cyanide inactivation of sulfite oxidase destroys the catalytic ability of the enzyme by modifying the molybdenum site, whereas electron paramagnetic resonance (EPR) and absorption studies on the cyanide-inactivated enzyme show that the b_5-like heme is fully oxidized and unmodified (Coughlan et al., 1980). The oxidation–reduction potentials of sulfite oxidase have been measured (Cramer et al., 1980) with the one-electron reduction potentials $E_{\mathrm{Mo(VI)/Mo(V)}} = +38$ mV and $E_{\mathrm{Fe(III)/Fe(II)}} = +84$ mV, at pH 7.0.

In order to investigate intramolecular electron transfer in sulfite oxidase, we have used laser flash photolysis to generate flavin semiquinones in situ which react with the oxidized redox sites of the enzyme (Cusanovich & Tollin, 1980; Ahmad et al., 1982; Tollin et al., 1982; Bhattacharyya et al., 1983). In such flash photolysis experiments, the concentration of flavin radical generated in a single flash is sufficiently smaller than that of oxidized sulfite oxidase so as to ensure that no single molecule of the enzyme is reduced by more than one electron and that pseudo-first-order conditions are obtained. This provides an excellent method to study the catalytically relevant rates of intramolecular electron transfer between the one-electron-reduced molybdenum and heme redox sites, as well as to characterize the interaction of sulfite oxidase with exogenous reductants. In the studies reported here, we have used the neutral semiquinone radical forms of lumiflavin (LFH·) and riboflavin (RFH·) ($E_{m,7} = -217$ mV; Draper & Ingraham, 1968) and the flavin-like molecule 5-deazariboflavin (dRFH·) ($E_{m,7} = -650$ mV; Blankenhorn, 1976), which provide low-potential reductants for interaction with the redox sites of sulfite oxidase. We describe below the results of such experiments with native and cyanide-inactivated holoenzyme and with the isolated heme peptide of sulfite oxidase.

MATERIALS AND METHODS

Sulfite oxidase was isolated from chicken liver according to the procedure of Kipke et al. (1988). The purified enzyme used in the experiments had heme to protein ratios (A_{413}:A_{280}) of 0.60 to 0.83. Analyses of representative enzyme preparations gave Mo:heme ratios of 1.03:1.00. The cyanide-inactivated enzyme was prepared as described by Coughlan et al. (1980) by treating the sulfite-reduced enzyme with sodium cyanide under anaerobic conditions followed by Sephadex G-25 gel filtration. The heme peptide was isolated according to the procedure of Guiard and Lederer (1977) by isolation of the chymotrypsin-digested enzyme using Sephadex G-75 chromatography. Sulfite oxidase activity was assayed as described by Cohen and Fridovich (1971a) by measuring the reduction of cytochrome *c* [0.2 M tris(hydroxymethyl)aminomethane (Tris), pH 8.5, with 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM cytochrome *c*, and 40 mM sodium sulfite]. The activity measurements were carried out at 25 °C using a Uvikon 810 spectrophotometer. The concentration of sulfite oxidase active centers was determined spectrophotometrically by using $\epsilon_{413\mathrm{nm}} = 99.9$ mM^{-1} cm^{-1} for the oxidized form as reported by Cohen and Fridovich (1971a). The concentration of the heme peptide was determined spectrophotometrically using this sulfite oxidase heme molar extinction coefficient.

All kinetic experiments were carried out at ambient temperature (23–25 °C) anaerobically in cuvettes sealed with serum stoppers which were deaerated by bubbling with water-saturated argon gas. The buffer used for the kinetic experiments was 20 mM potassium phosphate and 10 mM ethylenediaminetetraacetic acid (EDTA) with the appropriate flavin (≈70 μM) at pH 7.0. Laser photoexcitation was carried out with a nitrogen laser-pumped dye solution [2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene, $\lambda_{\max}$ 436 nm] for both lumiflavin and riboflavin, as previously described (Cusanovich & Tollin, 1980). For 5-deazariboflavin, the dye laser was a 1.2 mM solution of 1,4-bis(2-methylstyryl)benzene in *p*-dioxane as described previously (Bhattacharyya et al., 1983). The kinetic data were plotted according to the change in signal (ΔS) as a function of time. This is equivalent to a ΔA plot since the signal changes produced in the sample were small ($\Delta A < 0.01$). The MEDAS data analysis program (EMF Software, Baltimore, MD) was also used to fit some of the biphasic decay curves, with results comparable to those obtained by using hand-fitting.

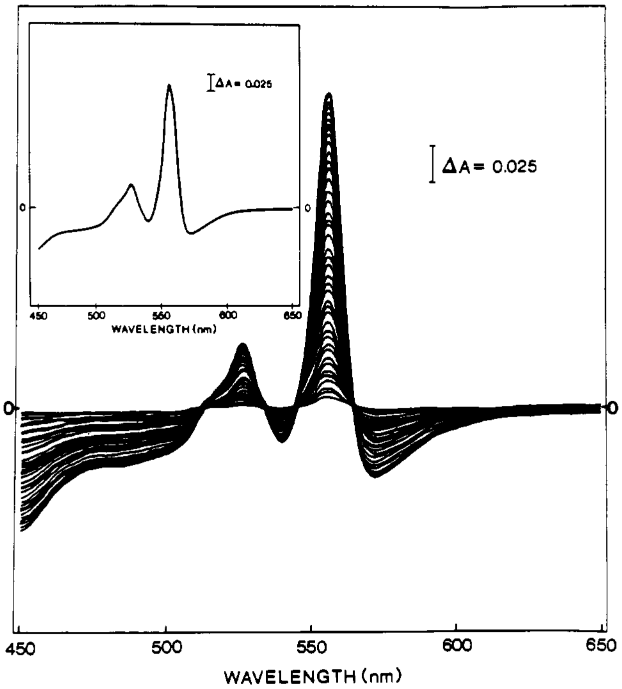


FIGURE 1: Phototitration difference spectrum (steady-state conditions) of sulfite oxidase using illumination from a microscope light at 20-s intervals. The solution contained 7 μM sulfite oxidase in the presence of 10 μM deazariboflavin under anaerobic conditions. The buffer used was 20 mM potassium phosphate, pH 7.0, with 1.0 mM EDTA. (Inset) Difference spectrum of sulfite oxidase, 4.6 μM, upon dithionite reduction under aerobic conditions. The buffer was 10 mM potassium phosphate, pH 7.0, with 0.10 mM EDTA.

RESULTS

Flash-Induced Difference Spectrum of the Native Enzyme. The visible spectrum of oxidized sulfite oxidase is dominated by the b_5-like heme prosthetic group of the enzyme. The spectrum of the molybdenum fragment from rat liver sulfite oxidase (Johnson & Rajagopalan, 1977) revealed a relatively weak absorbance for both oxidized [Mo(VI)] and reduced [Mo(V)] forms of the chromophore. The extinction coefficients for these absorbances are about an order of magnitude smaller than the values for the heme, and therefore, the molybdenum is not readily detectable in the intact enzyme by visible absorption spectroscopy. Thus, the change in absorbance at a fixed wavelength upon reduction of sulfite oxidase under steady-state conditions is dominated by the difference between the oxidized and reduced heme.

Steady-state photoreduction of sulfite oxidase with dRFH· using a microscope illuminator resulted in a difference spectrum equivalent to that obtained by dithionite reduction under similar conditions (Figure 1). However, the flash-induced difference spectrum of the enzyme obtained with dRFH· at

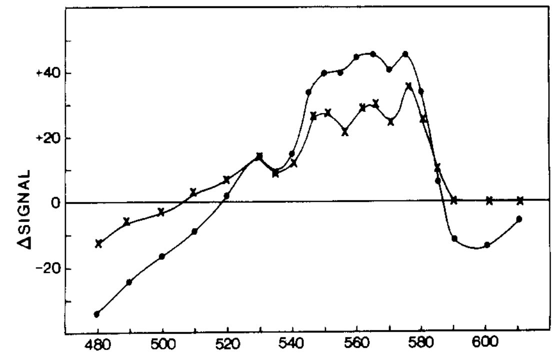


FIGURE 2: Flash-induced difference spectrum (oxidized minus reduced) observed at 3 ms (×) and 100 ms (●) after the laser flash for 10 μM sulfite oxidase in the presence of 100 μM 5-deazariboflavin. The buffer conditions were 20 mM potassium phosphate, pH 7.0, with 10 mM EDTA.

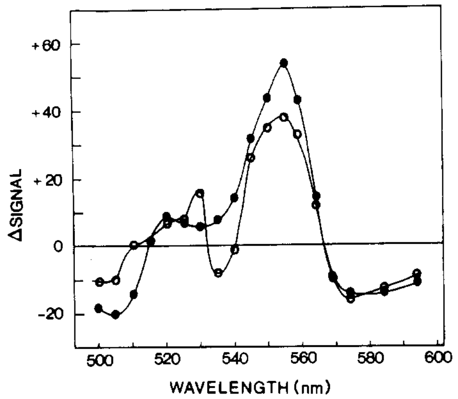


FIGURE 3: Flash-induced difference spectra (oxidized minus reduced) observed at 60 ms after the laser flash for 10 μM sulfite oxidase (●) and 20 μM sulfite oxidase heme peptide (○) in the presence of 47 μM lumiflavin. The buffer conditions were as described in Figure 2. The data were normalized at 585 nm.

3 and 100 ms after the laser flash (Figure 2) showed a relatively large positive absorbance in the spectral region 510–580 nm. This suggests that the reduced heme is overlayed by a transient species, as the steady-state difference spectra isosbestic points are lost. The positive absorbances which were observed under non-steady-state conditions have extinction coefficients similar to the α band of the heme and thus appear to be a different species than gives rise to the Mo(V) absorbance seen with the isolated molybdenum domain of the rat liver enzyme. The absorption changes in the region of the heme isosbestic points of the enzyme decayed to the preflash base line with a first-order rate constant of 0.18 s^{-1} (data not shown) and thus indicate the presence of a transient kinetic product.

The flash-induced difference spectrum of native sulfite oxidase obtained with LFH· was similar to the difference spectrum of the heme peptide under the same conditions (Figure 3), except for a small positive absorbance change underlying the heme α region of the spectrum. In contrast, the flash-induced difference spectrum of the heme peptide was essentially the same as the static difference spectra of both the native enzyme and the heme peptide. The flash-induced difference spectrum of the native enzyme gave a β (527 nm) to α (556 nm) ratio of 0.08, which is considerably less than the expected value of 0.17 obtained under steady-state conditions. The flash-induced difference spectrum of the heme peptide gave a β:α ratio of 0.16, which is in good agreement with the expected steady-state value. The difference spectra of the native enzyme and the heme peptide in Figure 3 were normalized to the kinetic data obtained at 585 nm, a wavelength which gave a sizable negative absorbance change upon reduction.

The additional positive absorbance changes seen in the flash-induced difference spectrum of the native enzyme using LFH· in the 530–560-nm spectral region are smaller in magnitude relative to the spectral features which can be associated with the heme. The additional positive spectral changes observed with both dRFH· and LFH· appear to involve the molybdenum site, because the flash-induced difference spectrum of the isolated heme peptide of sulfite oxidase (Figure 3) was similar to that obtained with both the native enzyme and the heme peptide of sulfite oxidase under steady-state conditions. It should be noted that, in the absence of added flavin, no detectable absorption changes were observed upon laser excitation of the sample.

Kinetic Studies of the Native Enzyme with Deazariboflavin. Two kinetically separable optical transients were observed at 470 nm following laser generation of dRFH· (Figure 4). Subsequent to the initial rapid increase in absorbance caused by the formation of the flavin radical by the laser flash (Edmondson et al., 1982), a decay was observed due to oxidation of the dRFH· (Figure 4A), with the absorbance going below the preflash base line as expected from both the flash-induced (Figure 2) and static difference spectra (Figure 1). This bleaching is clearly biphasic (Figure 4B). The fast phase

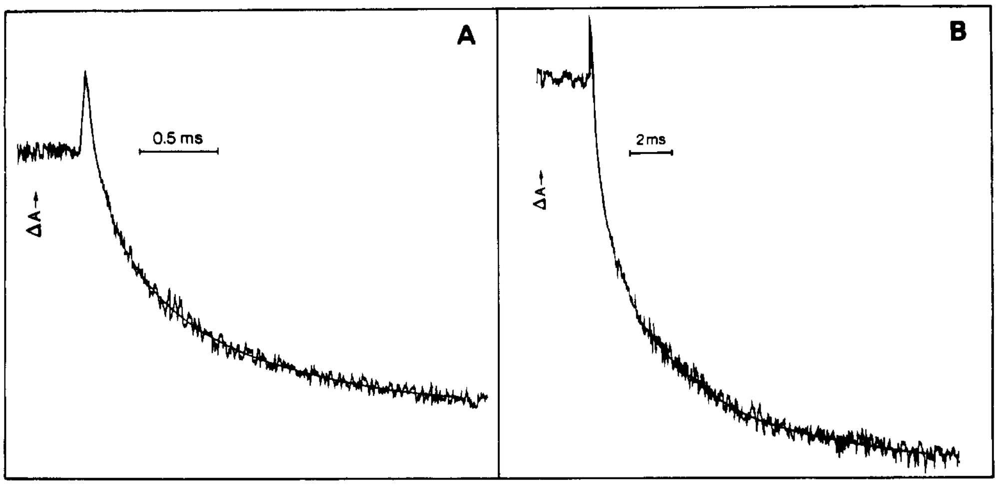


FIGURE 4: Transient absorbance change at 470 nm upon laser flash photolysis of 5 μM sulfite oxidase in the presence of 90 μM 5-deazariboflavin under anaerobic conditions. The buffer conditions were as described in Figure 2. (A) 3-ms time scale; (B) 20-ms time scale.

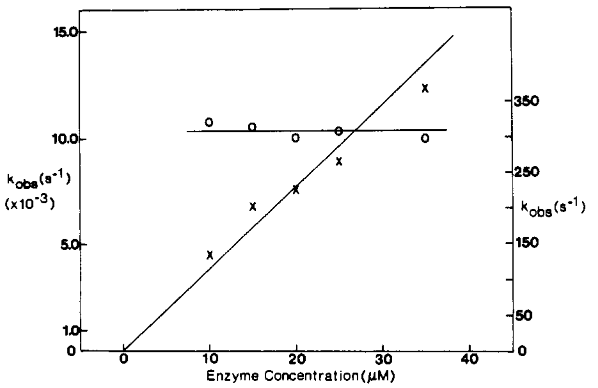


FIGURE 5: Dependence of k_{obs} for 5-deazariboflavin semiquinone decay upon sulfite oxidase concentration for the fast phase [(X) left y axis] and the slow phase [(O) right y axis]. Enzyme concentration is expressed in terms of molar heme concentration.

decay, which was calculated by subtracting the absorbance contributed by the slow phase from the total absorbance change, was resolved with short time sweeps (typically 1.5–3 ms; Figure 4A), and the slower process was determined by using longer time sweeps (15 ms; Figure 4B). In both cases, linear log Δ (signal) vs time plots were obtained (data not shown). The time-resolved difference spectra corresponding to these two kinetic phases are shown in Figure 2 (3- and 100-ms spectra). In order to elucidate the nature of the two kinetic processes, the concentration of the enzyme was varied. The observed rate constant for the fast phase showed a linear dependence on sulfite oxidase concentration with a calculated second-order rate constant of $(4.0 \pm 0.5) \times 10^8$ M^{-1} s^{-1} (Figure 5). In contrast, the observed rate constant for the slow phase was concentration independent with a first-order rate constant of 310 ± 11 s^{-1} (Figure 5) and accounted for approximately 50% of the total signal change observed upon laser flash reduction. The fast second-order reaction can be assigned to the direct reduction of the enzyme by dRFH·. Since a loss of absorbance occurs at 470 nm in the steady-state difference spectra, we presume that heme reduction is being monitored in the transient. The slow process also appeared as a bleach at this wavelength and is consistent with an intramolecular electron-transfer reaction, presumably occurring via molybdenum, generating additional reduced heme. This implies that the rate constant for direct molybdenum reduction in the native enzyme is $\geq(4.0 \pm 0.5) \times 10^8$ M^{-1} s^{-1}. On the basis of the thermodynamic driving force for reduction of the redox sites of the enzyme, intramolecular electron transfer from Mo(V) to Fe(III) would be expected to occur. The data shown in Figure 4B and another similar decay curve (15-ms time scale) were also fit by using MEDAS software and resulted in the following average rate constants and signal contributions: $k_1 = 4.1 \times 10^8$ M^{-1} s^{-1} (53%); $k_2 = 264$ s^{-1} (47%). Concentration dependence studies of the enzyme at various wavelengths showed that both the observed first- and second-order rate constants were wavelength independent.

Kinetic Studies of the Native Enzyme with Lumiflavin (LF) and Riboflavin (RF). LF and RF have identical reduction potentials ($E_{m,7} = -217$ mV) but differ in the substituent at the N(10) position of the flavin. If steric hindrance to reduction occurs, the bulky ribityl group of RFH· is expected to decrease the rate constant for electron transfer to a redox site relative to LFH· (Meyer et al., 1983). The kinetics of sulfite oxidase reduction were measured at 585 nm using photogenerated LFH· (Figure 6A) and RFH· (Figure 6B), because of the relatively large signal change observed at this wavelength upon reduction of the enzyme. The signals showed an initial fast monophasic absorbance change following radical formation which corresponded to heme reduction (Figure 3). With both LFH· and RFH·, a slower absorbance change was also observed which corresponded to heme reoxidation and which accounted for about 35% of the observed signal change (data at 550 nm using LFH· shown in Figure 6C). For LFH·, varying the enzyme concentration resulted in a second-order rate constant of $(2.9 \pm 0.2) \times 10^7$ M^{-1} s^{-1} for the fast phase, while for RFH· varying the enzyme concentration resulted in a second-order rate constant of $(1.6 \pm 0.3) \times 10^7$ M^{-1} s^{-1} for the fast phase (Figure 7). These second-order rate constants can be assigned to the direct reduction of the heme site by the flavin semiquinones. A steric hindrance to this reduction is apparent from the smaller rate constant obtained with RFH·. The first-order nature of the heme reoxidation process with LFH· (monitored at 550 nm) was established by varying the enzyme concentration. This resulted in a concentration-independent first-order rate constant of 155 ± 11 s^{-1} (Figure 7), consistent with intramolecular electron transfer to the Mo site. To test the possibility that the heme reoxidation process was due to traces of dissolved oxygen in the buffer, the enzyme sample (with flavin) was shaken in air for several minutes. Subsequent flash photolysis resulted in no change in the rate constant for heme reoxidation. Kinetic traces of the laser-reduced enzyme at 585 nm (60-ms time scale) using LFH· showed an increase in absorbance, subsequent to the second-order heme reduction process, which corresponded to the

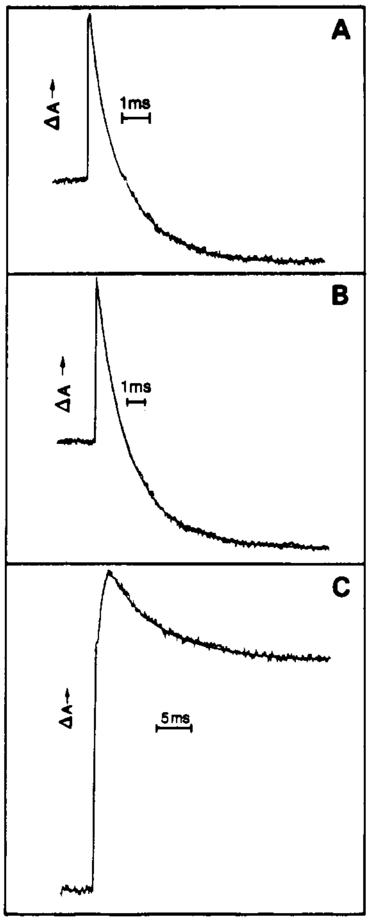


FIGURE 6: Transient absorbance changes upon laser flash photolysis of the enzyme under anaerobic conditions. The buffer conditions were as described in Figure 2. (A) 15 μM sulfite oxidase in the presence of 58 μM lumiflavin at 585 nm (10-ms time scale). (B) 15 μM sulfite oxidase in the presence of 78 μM riboflavin at 585 nm (15-ms time scale). (C) 20 μM sulfite oxidase in the presence of 58 μM lumiflavin at 550 nm (40-ms time scale).

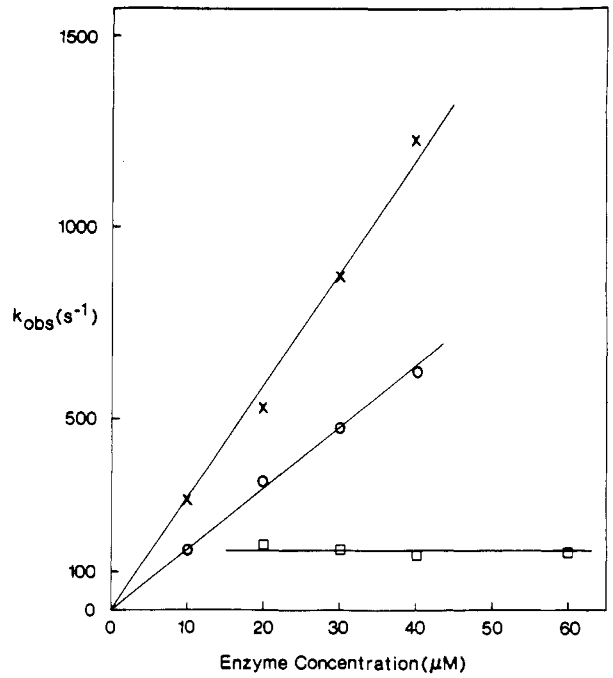


FIGURE 7: Dependence of k_{obs} for lumiflavin semiquinone decay for the fast phase (X) and the slow phase (□) upon sulfite oxidase concentration and the dependence of k_{obs} for riboflavin semiquinone decay for the fast phase (O) upon sulfite oxidase concentration. Enzyme concentration is expressed in terms of molar heme concentration.

first-order heme reoxidation process (data not shown). The kinetics of heme reoxidation were done at wavelengths where the transient signal was above the preflash base line in order to minimize the contribution from the heme reduction signal. The fact that intramolecular heme reduction was observed with dRFH· and intramolecular heme reoxidation was observed with LFH· (and RFH·) indicates that the primary reduced enzyme species formed upon laser photolysis was different for the two types of flavin. This is consistent with the observation that the flash-induced transient difference spectra were different for dRFH· and LFH· (Figures 2 and 3) and that substantially more of a molybdenum-associated transient was obtained with the former than with the latter. Furthermore, the observed first-order rate constant for heme reoxidation obtained with LFH· (155 ± 11 s^{-1}) was different from the observed first-order rate constant obtained from heme reduction using dRFH· (310 ± 11 s^{-1}). This implies that the chemical nature of the species involved in the intramolecular electron transfer between the Mo and heme sites is dependent upon the nature of the reductant.

Concentration dependence studies of the enzyme at various wavelengths using LFH· showed that both the observed second-order rate constant for heme reduction and the observed first-order rate constant for heme reoxidation were wavelength independent.

Kinetic Studies of Cyanide-Inactivated Enzyme. To confirm that the observed first-order rate constant for heme reduction obtained with dRFH· and the observed first-order rate constant for heme reoxidation with LFH· were correctly assigned to Mo(V) → Fe(III) and Fe(II) → Mo(VI) intramolecular electron transfer, respectively, we investigated the kinetics of the cyanide-inactivated enzyme under the same conditions as the native enzyme. It has been shown that treatment of reduced bovine and rat sulfite oxidase with cyanide results in a concomitant disappearance of the sulfite-generated Mo(V) EPR signal and a loss of catalytic ability, without effect on the absorption spectrum of the enzyme in the visible or near-ultraviolet regions (Cohen et al., 1971; Coughlan et al., 1980). We found that the chicken liver enzyme had the same properties following treatment of the reduced enzyme with cyanide.

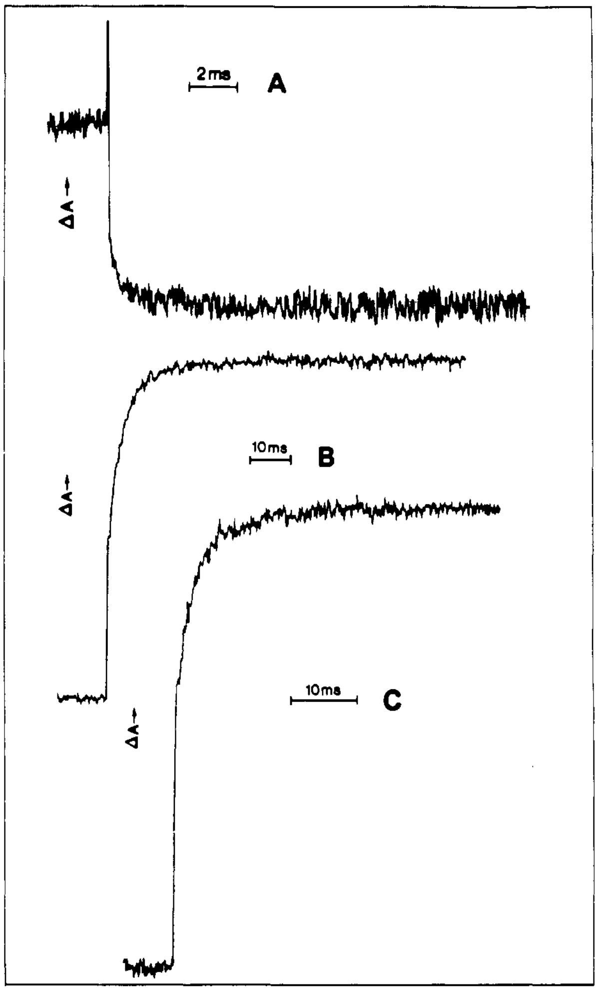


FIGURE 8: Transient absorbance changes upon laser flash photolysis of (A) 10 µM cyanide-inactivated sulfite oxidase at 470 nm in the presence of 90 µM 5-deazariboflavin (20-ms time scale), (B) 5 µM cyanide-inactivated sulfite oxidase at 565 nm in the presence of 48 µM lumiflavin (100-ms time scale), and (C) 20 µM sulfite oxidase heme peptide at 550 nm in the presence of 58 µM lumiflavin (100-ms time scale). The buffer conditions were as described in Figure 2.

Reaction of dRFH· with the cyanide-inactivated enzyme resulted in a fast bleaching at 470 nm, whereas the slow phase seen with the native enzyme was absent from the kinetic trace (Figure 8A; compare with Figure 4B). Varying the concentration of the enzyme gave a second-order rate constant of (3.9 ± 0.5) × 10^8 M^{-1} s^{-1}, which is the same within experimental error as the second-order rate constant for heme reduction obtained with the native enzyme. The absence of the slow phase is consistent with the hypothesis that cyanide inactivation of the molybdenum prevents reduction at this site and results in the absence of intramolecular electron transfer from Mo(V) to Fe(III).

Reaction of LFH· with the cyanide-inactivated enzyme again resulted in a rapid bleaching at 585 nm (not shown), whereas the heme reoxidation seen with the native enzyme was absent from the kinetic trace (Figure 8B; compare with Figure 6C). Varying the concentration of the enzyme gave a second-order rate constant of (2.7 ± 0.1) × 10^7 M^{-1} s^{-1} for heme reduction which was the same, within experimental error, as the second-order rate constant obtained for heme reduction with the native enzyme. The absence of heme reoxidation with the cyanide-inactivated enzyme using LFH· is consistent with the hypothesis that the Fe(II) → Mo(VI) intramolecular electron-transfer process seen with the native enzyme is not present due to inactivation of the molybdenum site.

Although treatment of the enzyme with cyanide results in a catalytically inactive Mo site, this would not necessarily prevent reduction of molybdenum by the photogenerated flavin radicals. Thus, for the cyanide-inactivated enzyme, the absence of the observed first-order heme reduction seen with the native enzyme using dRFH· and the absence of the observed first-order heme reoxidation seen with the native enzyme using LFH· could simply mean that the redox potential of the cyanide-treated Mo site is shifted to a considerably negative value relative to the native enzyme. This is consistent with the observation (Coughlan et al., 1980) that the molybdenum center in the cyanide-inactivated enzyme exists in an oxidation state lower than that obtained following the oxo-transfer reaction during catalysis [Mo(IV)]. In addition, the observation that the second-order rate constants for heme reduction of the cyanide-inactivated enzyme were the same as those for heme reduction of the native enzyme indicates that cyanide inactivation does not alter the heme site. This parallels the observation that the static visible absorption spectrum of the cyanide-inactivated enzyme is unchanged relative to the visible absorption spectrum of the native enzyme.

Kinetic Studies of the Sulfite Oxidase Heme Peptide. Further confirmation of the assignments of the observed first-order rate processes seen with the native enzyme as being due to intramolecular electron transfer between the molybdenum and heme redox sites was obtained by flash photolysis studies of the isolated heme peptide of sulfite oxidase. To date, efforts to obtain both the molybdenum and heme domains of sulfite oxidase have succeeded only with the rat liver enzyme (Johnson & Rajagopalan, 1977). Chymotryptic digestion of chicken liver sulfite oxidase resulted in the isolation of the heme domain (M_r 11-K) as the first species eluting from a G-75 column, and this species was without a molybdenum domain as shown by monitoring the fractions for ferricyanide reduction upon addition of sulfite (Guiard & Lederer, 1977). The absorption spectra of the oxidized and reduced heme peptide were identical with those of the native enzyme.

Kinetic studies with the heme peptide using dRFH· showed both fast and slow pseudo-first-order rates for reduction of the b_5-type heme. However, in contrast to the native enzyme, varying the concentration of the heme peptide indicated that both processes were second order. A rate constant of $(2.9 \pm 0.2) \times 10^8$ M^{-1} s^{-1} (data not shown) was obtained for the fast process, which is smaller than that for the fast second-order reduction of heme in the native protein [$(4.0 \pm 0.5) \times 10^8$ M^{-1} s^{-1}], whereas the slower process had a second-order rate constant of $(1.5 \pm 0.1) \times 10^7$ M^{-1} s^{-1} (data not shown). No first-order processes were detected, again indicating that the first-order process observed with the native enzyme involves the molybdenum site. The fast process accounted for 65% of the total signal change, while the slow process accounted for 35% of the total signal change. The kinetics of reduction of the heme peptide by LFH· were monophasic. The second-order rate constant obtained from a concentration study was $(1.6 \pm 0.1) \times 10^7$ M^{-1} s^{-1} (data not shown), which is again smaller than the second-order rate constant obtained for heme reduction of the native enzyme. The absence of the observed first-order reoxidation process seen with the native enzyme using LFH· (Figure 8C; compare with Figure 6C) indicates that the heme reoxidation reaction involves the molybdenum site.

Table I: Rate Constants Obtained with the Native Enzyme, Cyanide-Inactivated Enzyme, and the Heme Peptide Using the Reductants dRFH·, LFH·, and RFH·[a]

	reductant		
enzyme form	dRFH·	LFH·	RFH·
native	$(4.0 \pm 0.5) \times 10^8$ M^{-1} s^{-1} 310 ± 11 s^{-1}	$(2.9 \pm 0.2) \times 10^7$ M^{-1} s^{-1} 155 ± 11 s^{-1}	$(1.6 \pm 0.3) \times 10^7$ M^{-1} s^{-1}
cyanide inactivated	$(3.9 \pm 0.5) \times 10^8$ M^{-1} s^{-1}	$(2.7 \pm 0.1) \times 10^7$ M^{-1} s^{-1}	
heme peptide	$(2.9 \pm 0.2) \times 10^8$ M^{-1} s^{-1} $(1.5 \pm 0.1) \times 10^7$ M^{-1} s^{-1}	$(1.6 \pm 0.1) \times 10^7$ M^{-1} s^{-1}	

[a] All second-order rate constants are expressed in terms of molar heme concentration.

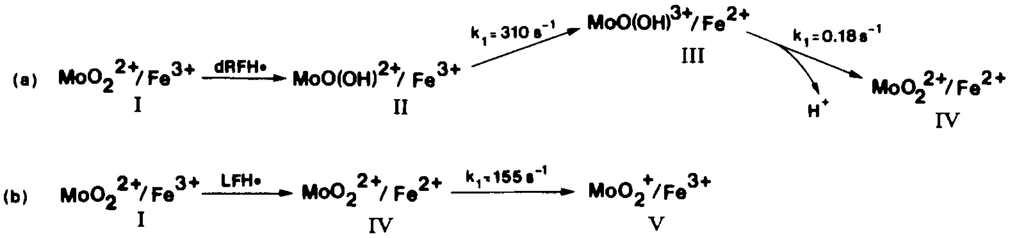
The results of these kinetic studies suggest that a structural alteration of the heme subunit by chymotryptic digestion of the native enzyme has occurred, as evidenced by the biphasic second-order kinetics with dRFH· and the reduced rates of reduction compared with the holoenzyme. The reduction potential for the cleaved heme subunit of beef liver sulfite oxidase has also been reported to be decreased relative to the value obtained with the native enzyme at pH 7 (Cramer et al., 1980). The additional second-order rate constants seen with dRFH· but not with LFH· suggest the formation of a low-potential form of the heme peptide which can be reduced only by the more strongly reducing deazariboflavin radical. The rate constants obtained with the native enzyme, the cyanide-inactivated enzyme, and the isolated heme peptide are summarized in Table I.

DISCUSSION

The laser flash photolysis experiments described above clearly demonstrate that two distinctly different intramolecular electron-transfer processes involving Mo and heme occur in sulfite oxidase when either deazaflavin or ordinary flavin semiquinone is used as the reductant. With dRFH· a second-order reduction of the heme site was followed by a slower first-order heme reduction corresponding to intramolecular electron transfer from Mo(V) to Fe(III). LFH· and RFH· also reduced the heme site in a second-order manner, but this was followed by a first-order reoxidation of the heme due to intramolecular electron transfer from Fe(II) to Mo(VI). These assignments are supported by flash photolysis studies of modified sulfite oxidase. The different directions of the observed intramolecular electron-transfer processes in the enzyme using dRFH· and LFH· can be interpreted in terms of the chemical and thermodynamic differences between these two types of flavins. The results using dRFH· indicate that the Mo site was reduced more readily than the heme site ($\geq 4.0 \times 10^8$ M^{-1} s^{-1}) with this reductant whereas the heme site was reduced more readily than the Mo site using LFH· as the reductant. This is inconsistent with the expected second-order rate constants for reduction of the redox sites based on the thermodynamic driving force, which predict that with both dRFH· and LFH· heme reduction would be favored (Meyer et al., 1983).

5-Deazariboflavin has a central pyridine ring rather than the pyrazine ring present in lumiflavin, and whereas the latter acts as a reductant by transferring an electron (Draper et al., 1968), dRFH· may be able to act as a reductant by both one-electron transfer and hydrogen atom transfer. All of our kinetic results indicate that the heme site of the enzyme is not involved in any chemical reactions with the reductants other

Scheme I: Proposed Reactions of the Chemical Reductants dRFH· (a) and LFH· (b) with the Redox Sites of Sulfite Oxidase



than direct one-electron transfer. On the other hand, it is possible to rationalize our observations by assuming that the Mo site of the enzyme is responsible for the different chemical reactivities of the reductants.

Extended X-ray absorption fine structure (EXAFS) data for sulfite oxidase indicate that there are two oxo atoms in the oxidized form of the enzyme, whereas in the dithionite-reduced form only a single terminal oxo atom is present with the other oxygen atom presumably protonated to the hydroxyl form (Cramer et al., 1979, 1981). Data obtained with the EPR-active Mo(V) state of the enzyme support this observation at low pH (about pH 6) inasmuch as superhyperfine coupling is observed in the molybdenum spectrum due to interaction with a proton, whereas at high pH (about pH 9) the superhyperfine splitting is no longer present (Kessler & Rajagopalan, 1972; Lamy et al., 1980). These low-pH and high-pH species may arise from a $[MoO(OH)]^{2+}$ core and a $[MoO]^{3+}$ or $[MoO_2]^+$ core, respectively (Lamy et al., 1980; Cramer, 1983; Bray et al., 1983). The interconversion of the species depends upon both pH and chloride concentration (Bray et al., 1983). However, recent data examining the proton spin-flip transitions in the Mo(V) EPR of sulfite oxidase indicate a coupled, exchangeable proton at high pH which is suggested to arise from an MoOH group (George, 1985) and imply that a $[MoO(OH)]^{2+}$ core is present in both the high-pH and low-pH forms of the enzyme. Our laser-induced photoreduction kinetics of sulfite oxidase at pH 7 by dRFH· and LFH· are consistent with these reductants respectively forming the $[MoO(OH)]^{2+}$ and $[MoO_2]^+$ cores (vide infra).

An equilibrium exists between the high-pH and low-pH forms of the Mo(V) state at pH 7 as evidenced by EPR (Lamy et al., 1980). The oxidation–reduction potentials for the Mo(VI)/Mo(V) and Fe(III)/Fe(II) couples of sulfite oxidase have been measured both at pH 7 and at pH 9. While the heme potential has been found to be largely pH independent, the molybdenum (VI/V) potential shifts to a lower value when the pH is raised (Cramer et al., 1980). This is expected for a site with a dissociable proton and indicates that at low pH the Mo(VI) center is more easily reduced, supporting the data which show that proton uptake is associated with reduction of the molybdenum center (Harris & Hellerman, 1956). Thus, the reported midpoint potential for the Mo(VI)/Mo(V) couple at pH 7 is for a mixture of the low-pH and high-pH forms of Mo(V) and would be expected to shift to a more positive value as the pH is lowered. The one-electron reduction of the oxidized molybdenum site, $[MoO_2]^{2+}$, to the $[MoO(OH)]^{2+}$ species should proceed at a redox potential more positive than the published value at pH 7, and the reduction of $[MoO_2]^{2+}$ to $[MoO_2]^+$ would have a redox potential more negative than the published value at pH 7. The heme potential would be essentially unaltered.

The ability of dRFH· to function as a reductant by transferring a hydrogen atom could produce a molybdenum site with a more positive reduction potential, due to the formation of the $[MoO(OH)]^{2+}$ species, and may account for the larger degree of Mo reduction. Conversely, because LFH· functions as a reductant by transferring an electron, a $[MoO_2]^+$ species would result, and the reduction potential of the Mo site would shift to a more negative value, resulting in heme reduction being a more favorable process. Scheme I represents the proposed interaction of the reductants dRFH· and LFH· with sulfite oxidase. Reduction of the enzyme with dRFH· (reaction sequence a) results in the production of forms II and III, with the ratio of II/III being greater than that expected from the equilibrium constant value. This leads to intramolecular electron transfer primarily from the one-electron-reduced Mo site to the oxidized heme site in form III. The formation of the final reduced heme species (form IV) would require the loss of a proton, and this is presumably the slow first-order bleach (0.18 s^{-1}) noted above. Reduction of the enzyme with LFH· (reaction sequence b) results in the production of forms IV and V, with the ratio of IV/V being greater than that expected from the equilibrium constant value. This results in intramolecular electron transfer primarily from the reduced heme site to the oxidized Mo site in form IV. Thus, this scheme can account for both the different directions of intramolecular electron transfer and the different values for the first-order rate constants.

Support for the hypothesized low-pH and high-pH forms of sulfite oxidase and confirmation of the expected relative redox potentials of these species have been recently obtained by electrochemical generation of $[MoO(OH)]^{2+}$ and $[MoO_2]^{2+}$ using model compounds (Farchione et al., 1986; Hinshaw & Spence, 1986; Dowerah et al., 1987). Electrochemical data for the one-electron reduction of $[MoO_2]^{2+}$ give redox potential values for the generation of the dioxo-Mo(V) species but show that in the presence of a proton source the $[MoO(OH)]^{2+}$ core is rapidly formed (Farchione et al., 1986; Hinshaw & Spence, 1986; Dowerah et al., 1987). Electrochemical reduction of $[MoO_2]^{2+}$ in the presence of a proton source (Dowerah et al., 1987) results in a reduction potential that is about 200 mV more positive than the reduction potential obtained in the absence of a proton source. Although the protonated species has not been characterized, the positive shift in the value of the redox potential is consistent with the pH-dependent shift in the redox potential of the enzyme.

The flash-induced difference spectra of the native enzyme using dRFH. (Figure 2) and LFH· (Figure 3) showed absorbances in the α-band region of the heme spectrum that are not present in the steady-state difference spectra of the native enzyme or the heme peptide. The evidence presented above suggests that these electronic transitions are associated with the molybdenum site. Furthermore, charge transfer transitions are indicated because these changes have comparable intensities to the reduced heme α band at 556 nm. According to Scheme I, this would occur by using dRFH· as a consequence of the formation of forms II and III. Since a different species would be produced by using LFH·, form V, the different spectral properties of the transients (Figure 2 and 3) would be accounted for. Electronic spectral studies of molybde-

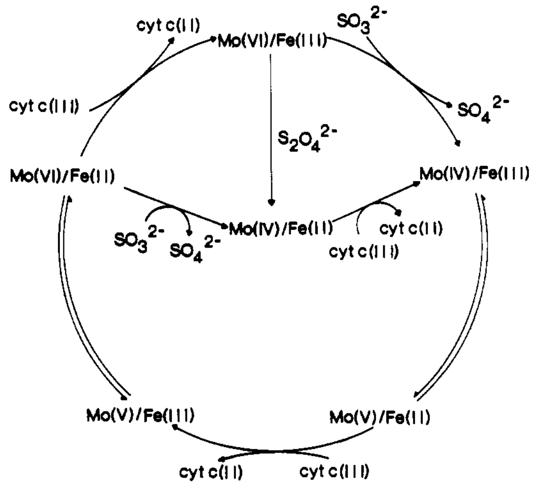


FIGURE 9: Proposed catalytic cycle of sulfite oxidase. The equilibrium process between the Mo(VI)/Fe(II) and Mo(V)/Fe(III) states of the enzyme corresponds to the portion of the cycle studied by flash photolysis.

num(VI) complexes with the $[MoO]^{4+}$ core (Wilshire et al., 1980; McCleverty, 1968) indicate that these complexes can be intensely colored whereas, in contrast, molybdenum(VI) complexes with the $[MoO_2]^{2+}$ core typically exhibited absorbances in the 250–400-nm region and are not highly colored (Stiefel, 1977). Molybdenum(V) complexes with the $[MoO]^{3+}$ core (Cleland et al., 1987) and the $[MoO_2]^+$ core (Dowerah et al., 1987) have also been shown to absorb visible light. With dRFH·, forms II and III in Scheme Ia would be colored and contribute to the absorbances seen in the time-resolved difference spectra at 3 and 100 ms, respectively (Figure 2). The loss of a proton at the rate of 0.18 s^{-1} would result in form IV (Scheme Ia) and account for the bleaching of the transient signals in the α-band region of the heme spectrum as a result of forming the presumably colorless $[MoO_2]^{2+}$ core. With LFH·, form V would be responsible for the transient absorbance in the α region of the heme spectrum upon flash photolysis.

Bray and co-workers (Bray et al., 1983) reported that the Mo(V) EPR spectrum of the enzyme in phosphate buffer produced a signal that was distinctly different from both the high-pH and low-pH signals and proposed a phosphate-bound Mo(V) species. Therefore, flash photolysis kinetics of the enzyme were also investigated in 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.0) with 10 mM EDTA. The results were qualitatively similar to the results obtained in phosphate buffer. When dRFH· was used, a second-order reduction of the heme site was observed followed by a first-order heme reduction process [Mo(V) → Fe(III)], whereas when LFH· was used, a second-order reduction of the heme site was followed by a first-order heme reoxidation process [Fe(II) → Mo(VI)]. However, the relative ratios of the fast/slow processes using the two photoreductants were considerably different from the ratios obtained in phosphate buffer. Laser photolysis using dRFH· resulted in a fast:slow ratio of 80:20 (compared to 50:50 in phosphate buffer), whereas a fast:slow ratio of 35:65 was obtained by using LFH· (compared to 65:35 in phosphate buffer). This would suggest that the Mo(VI)/Mo(V) and/or Fe(III)/Fe(II) reduction potentials of the enzyme have shifted in Hepes buffer relative to the reduction potentials in phosphate buffer. Because the kinetic time scale of the flash photolysis experiment is considerably faster than the time scale used to generate the Mo(V) EPR signal in phosphate buffer, the factor(s) responsible for the change in kinetic behavior of the enzyme in phosphate buffer relative to Hepes buffer cannot be determined. However, it is important to emphasize that regardless of whether phosphate buffer or Hepes buffer is used in the flash photolysis experiment, the direction of intramolecular electron transfer remains unaltered: i.e., Mo(V) → Fe(III) using dRFH· and Fe(II) → Mo(VI) using LFH·. This supports the hypothesis that the direction of intramolecular electron transfer depends upon the difference between dRFH· and LFH· as reductants and is consistent with Scheme I.

It has been proposed that the Mo is coordinated to a side chain of the pterin-containing molybdenum cofactor in sulfite oxidase (Johnson & Rajagopalan, 1982). Therefore, the possibility that reduction of the enzyme using dRFH· occurs at the pterin rather than the Mo site cannot be overlooked. The reduced pterin moiety could then presumably transfer an electron intramolecularly to the heme site. The additional absorbances seen in the flash-induced difference spectra (Figure 2) could be due to charge transfer transitions from such a reduced pterin to the molybdenum atom.

The catalytic cycle of sulfite oxidase (Rajagopalan, 1980; Garner et al., 1982; Figure 9) shows the oxo-transfer reaction and the electron-transfer processes that regenerate the fully oxidized, catalytically active enzyme. The intramolecular electron-transfer rates of 310 and 155 s^{-1} obtained by flash photolysis correspond to the equilibrium between the Mo(VI)/Fe(II) and Mo(V)/Fe(III) states of the enzyme and can be compared to the k_{cat} value of 29 s^{-1} we observe under the same conditions. However, because the molybdenum ligation character of sulfite oxidase and that of the enzymatic species formed during flash photolysis are not known, it is not possible to determine if our results reflect actual catalytic processes.

ACKNOWLEDGMENTS

We thank Kathleen Gibson for help in purifying enzyme for these studies and Dr. K. V. Rajagopalan for helpful suggestions concerning the properties and stability of the chicken liver enzyme.

Registry No. dRFH·, 78548-68-2; LFH·, 34533-61-4; RFH·, 35919-91-6; Mo, 7439-98-7; heme, 14875-96-8; sulfite oxidase, 9029-38-3.

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