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Enzymatic Redox Chemistry: A Proposed Reaction Pathway for the Six-Electron Reduction of SO_3^{2-} to S^{2-} by the Assimilatory-Type Sulfite Reductase from *Desulfovibrio vulgaris* (Hildenborough)†

Jian Tan and J. A. Cowan*
Evans Laboratory of Chemistry, The Ohio State University, 120 West 18th Avenue, Columbus, Ohio 43210
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ABSTRACT: A detailed reaction pathway for the six-electron reduction of SO_3^{2-} to S^{2-} by the assimilatory-type sulfite reductase (SiR) from *Desulfovibrio vulgaris* (Hildenborough) has been duduced from experiments with ^{35}S-labeled enzyme and the relative reaction rates of nitrogenous subtrates. The ligand bridging the prosthetic $[Fe_4S_4]$-siroheme center is apparently exchanged by $^{35}S^{2-}$ in both oxidized and reduced enzyme. This $^{35}S^{2-}$ label was retained in the course of SO_3^{2-} reduction, implicating substrate binding to the nonbridging axial site of the siroheme. A reaction mechanism is proposed in which SO_3^{2-} binds to Fe^{2+} through the sulfur atom, followed by a series of two-electron reductive cleavages of S-O bonds. Protonation of oxygen facilitates bond cleavage, giving hydroxide as leaving group. The bridge remains intact throughout the course of the reaction, providing an efficient coupling pathway for electron transfer between the cluster and siroheme.

The understanding of enzymatic reduction of low molecular weight substrates (e.g., NO_3^-, NO_2^-, SO_4^{2-}, SO_3^{2-}, O_2, N_2) is of general importance and has been widely studied (Mortenson & Thorneley, 1979; Barber, 1984; Witt et al., 1986; Papa, 1983; Hatefi et al., 1985). However, the molecular mechanisms for these reaction pathways are currently lacking in detail. The biological sulfur cycle provides many examples of this type of chemistry. Several sulfate- and sulfite-reducing systems have been described that either provide a source of reduced sulfur for cellular metabolism (assimilatory reduction) or have involvement in anaerobic respiration (dissimilatory reduction) (Le Gall & Postgate, 1973; Peck & Le Gall, 1982; Siegel, 1975). Reduction of SO_4^{2-} to SO_3^{2-} via an adenosyl phosphosulfate intermediate is catalyzed by a flavoprotein (ATP sulfurylase) and adenylyl phosphosulfate reductase (Le Gall & Postgate, 1973; Peck & Le Gall, 1982; Siegel, 1975). Subsequent six-electron reduction of SO_3^{2-} to S^{2-} is catalyzed by a single enzyme where the siroheme chromophore is re-

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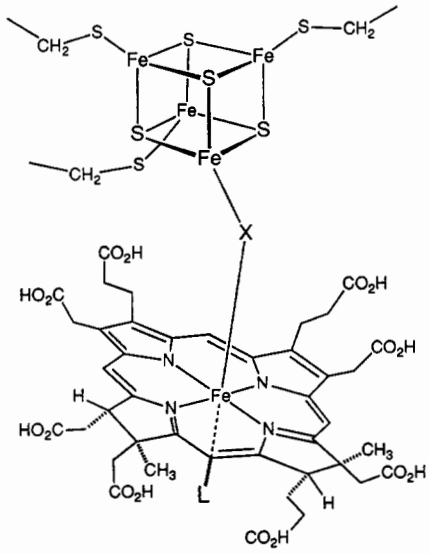
Chart I[a]



[a] X denotes the bridging ligand. Site L is unoccupied in *E. coli* sulfite reductase.

quired for sulfite binding (Young & Siegel, 1988). The heme subunit of the NADPH–sulfite reductase from *Escherichia coli* (M_r ~57 000) is the most extensively characterized enzyme in this class of redox proteins (Siegel et al., 1973, 1982; Christner et al., 1984; Siegel & Davis, 1974; Murphy et al., 1973; Janick et al., 1983; Janick & Siegel, 1983; Wilkerson et al., 1983). The proposed active site is the generic exchange-coupled $[Fe_4S_4]$–siroheme complex shown in Chart I (Krueger & Siegel, 1982; Cline et al., 1986; Christner et al., 1983, 1984; Madden et al., 1989; McRee et al., 1986) that has been postulated in most sulfite-reducing enzymes (Huynh et al., 1984; Moura et al., 1986) and several nitrite reductases (Vega & Kamin, 1977; Lancaster et al., 1979; Murphy et al., 1974; Crowe et al., 1983a,b).

Model studies on synthetic complexes have demonstrated that pentacoordinate ferric isobacteriochlorins are high spin, whereas hexacoordinate species are low spin (Stolzenberg et al., 1981). Recently a number of low molecular weight (M_r ~27 000) assimilatory sulfite reductases have been isolated from three anaerobic bacteria [*Methanosarcina barkeri*, *Desulfuromonas acetoxidans*, and *Desulfovibrio vulgaris* (Hildenborough)] that possess low-spin sirohemes on the basis of Mossbauer and EPR data (Huynh et al., 1984; Moura et al., 1986) and are therefore distinguished from the high-spin pentacoordinate center in the *E. coli* enzyme. Our experiments have focussed on the *D. vulgaris* enzyme (Huynh et al., 1984). Recent NMR studies suggest histidine as the sixth ligand (Cowan & Sola, 1990). The assignment of histidine ligation rather than adventitious coordination by S^{2-} during protein isolation is supported by comparison of published EPR *g*-values for low spin siroheme in native sulfite reductase (*D. vulgaris*) (g = 2.44, 2.36, 1.77) (Huynh et al., 1984), and the CN^- (g = 2.39, 2.33, 1.67) and S^{2-} (g = 2.24, 2.21, 1.96) adducts of the *E. coli* enzyme (Janick & Siegel, 1983). Analysis of the g-tensor elements for the low-spin hemes by the procedure of Blumberg and Peisach (Blumberg & Peisach, 1971; Palmer, 1986; Tan & Cowan, 1990) gives a measure of the rhombicity and tetragonal field splitting (TFS) for the ligand environment that correlates with the strength and asymmetry of the two apical ligands bound to the siroheme center. The utility of the method has been demonstrated by analysis of EPR parameters from a number of cytochromes, myoglobins, hemoglobins, and model systems (Blumberg & Peisach, 1971; Palmer, 1986). Clearly the $g_{x,y,z}$ components of the *D. vulgaris* reductase are more closely related to the CN^- adduct of the *E. coli* enzyme than those of the S^{2-} adduct. Previous measurements on heme adducts show that the rhombicity and TFS are similar for histidine and CN^- complexes (Blumberg & Peisach, 1971; Palmer, 1986), supporting coordination by histidine as deduced by NMR.

With regard to the bridging ligand, Peck and co-workers have suggested inorganic sulfide (Huynh et al., 1984) in the *D. vulgaris* reductase on the basis of chemical analysis data, in contrast to the cysteine thiolate bridge proposed for the *E. coli* enzyme (Madden et al., 1989; McRee et al., 1986; Ostrowski et al., 1989). The likelihood of a sulfide bridge in the former is supported by $^{35}S^{2-}$-exchange experiments described herein. An important conclusion that will emerge from the discussion below, however, is that the identity of the bridge unit is unlikely to be important with regard to the details of enzymatic reaction chemistry. The bridge serves only to electronically connect the two prosthetic centers.

Despite the apparent differences in the coordination environment of the siroheme in the sulfite reductases from *E. coli* and *D. vulgaris*, the overall structural arrangement adopted by the prosthetic groups is likely to be general. In this regard, Pierik and Hagen (1991) have proposed a distinct model for the active site in sulfite-reducing enzymes that is based on EPR studies of desulfoviridin, an abundant dissimilatory sulfite reductase found in *D. vulgaris*. The possibility of uncoupled centers and the presence of $[Fe_6S_6]$ clusters was discussed. While this is not in accord with NMR (Cowan & Sola, 1990) or Mossbauer (Huynh et al., 1984) data on the assimilatory reductase from *D. vulgaris*, it is clear that this general class of enzyme deserves further attention.

Despite the inherent potential of low molecular weight sulfite reductase enzymes as a vehicle for understanding biological oxidation–reduction reactions, no work has been reported on the molecular details of the reaction pathway. Accordingly, we have addressed three questions that pertain to the reaction mechanism of assimilatory sulfite reduction in *D. vulgaris*: (1) On which face of the siroheme does sulfite bind? (2) Does turnover proceed via Fe–S or Fe–O coordination? (3) What is the probable identity of intermediates formed along the reaction pathway? It is likely that many details of the reaction pathway will be generally valid for related enzymes. Although putative reaction schemes have been outlined previously in broad detail (Reynolds & Holm, 1989; Scheidt et al., 1988), our results provide evidence in support of a specific mechanism.

In this paper we demonstrate that the assimilatory sulfite reductase from *D. vulgaris* affords a convenient model for understanding the details of complex multielectron redox chemistry in biology. It is accessible to study by both high-field ^1H NMR and direct electrochemistry (unpublished results), while we have also recently cloned the reductase gene (Tan et al., 1991). Herein we describe experiments aimed at elucidating the reaction chemistry and propose a working model for the catalytic mechanism.

Materials and Methods

Materials and General Equipment. Radiolabeled $Na_2{}^{35}S{\cdot}9H_2O$ (14 mCi/mmol) was obtained from American Radiolabeled Chemicals Inc. Solid $Na_2{}^{35}S{\cdot}9H_2O$ (23.6 mg) was dissolved in H_2O (1 mL) to give a 0.1 M solution, which was stored at −20 °C. Elemental sulfur-34 (90% enrichment) was obtained from U.S. Services Inc. Reduction to $H_2{}^{34}S$ was carried out by heating $^{34}S(s)$ (50 mg) over a H_2 atmosphere. Two bubblers were connected in series; the first contained $^{34}S(s)$, and the second contained 0.1 M NaOH (1 mL) to trap

the product gas. Elemental $^{34}S(s)$ was heated with a Bunsen flame while the system was purged with $H_2(g)$. The best yields obtained were ca. 30%. Concentrations of sulfide stock solutions were accurately determined by a spectrophotometric assay (the methylene blue reaction) (Fogo & Popowsky, 1949; Siegel, 1965; Cammack et al., 1978) and were found to decrease slowly with time as a result of oxidation to thiosulfate, which was quantitated by use of Sorbo's test (Sorbo, 1957). Scintillation counting was carried out on a Beckman LS7000 instrument using Ecolite (+) scintillation fluid (ICN Radiochemicals). Buffer salts were of molecular biology grade (Fisher). Measurements of solution pH were carried out with an Accumet pH Meter 910 equipped with a Fisher gel-filled combination pH electrode. Sephadex G-100 gel-filtration material was obtained from Sigma, and DE-52 and CM-52 ion-exchange resins were from Whatman. Deionized water was used in all procedures and was obtained by filtering distilled water through a Barnstead nanopure system. Electronic absorption spectra were measured on a Perkin-Elmer λ 6 spectrophotometer that was interfaced to an Epson Equity I-plus computer.

Culture Growth/Protein Isolation and Purification. D. vulgaris (Hildenborough, NCIB 8303) was grown in an enriched Baars medium (ATCC medium no. 1249) at the Fermentation Laboratory, Department of Biochemistry, University of Wisconsin. The isolation and purification of the assimilatory sulfite reductase followed the procedure of Peck and co-workers (Huynh et al., 1984). Cytochrome c_3 and the large cytochrome used in control experiments were also isolated from *D. vulgaris* (Tan & Cowan, 1990). Other proteins used in control experiments [sperm whale myoglobin and high-potential iron protein (*Chromatium vinosum*)] were conveniently at hand in our laboratory and were purified following established procedures (Axup et al., 1988; Bartsch, 1971, 1978).

Kinetic Data. Kinetic data was obtained at 295 K by monitoring the decrease in absorbance at 600 nm from the $MeV^{+\bullet}$ radical anion used as an electron source for the enzyme during turnover. The enzyme (10 μL of a 15.8 μM stock) and substrate (50 μL of a 0.1 M stock) were added to a septum-stoppered quartz glass cell and degassed by repeated (5 cycles) evacuation and purging with oxygen-free nitrogen (prepurified nitrogen was passed through a radox scrubbing tower) on a double-manifold vacuum line. To this was added 0.96 mL of a previously prepared $MeV^{+\bullet}$ solution (zinc-reduced MeV^{2+}: $[MeV^{+\bullet}] = 40$ mM, $\epsilon_{600nm} = 1.3 \times 10^4$ cm^{-1} M^{-1}; Thorneley, 1974) that had been degassed as described above and the rate of change of the absorbance monitored at 600 nm. The resulting decay profile was hand digitized and a ln (abs) vs time plot was fitted by use of a standard nonlinear least-squares routine on a Macintosh computer.

Incorporation of $^{35}S^{2-}$ and Isolation of Radiolabeled Enzyme SiR($^{35}S^{2-}$). The exact conditions of each experiment are described in the Results section. In general, a 4–20-fold excess of $^{35}S^{2-}$ was added to a solution containing 9.5 nmol of enzyme. After incubation for 20 min to 1 h, the mixture (50–150 μL) was loaded onto the gel-filtration column [21 × 0.8 cm of Sephadex G-100 equilibrated with 50 mM potassium phosphate buffer (pH 7.6)] to separate protein from free anion. The column was then eluted with the equilibration buffer (ca. 20 mL). The eluent was collected in approximately equal fractions (1 mL), and a sample (5 μL) from each fraction was added to scintillation fluid (5 mL) prior to counting. These procedures were employed for sulfite reductase, high-potential iron protein (HiPIP), cytochrome c_3, the large cytochrome, and sperm whale myoglobin (Mb). Reduced SiR was obtained by adding $MeV^{+\bullet}$ (10–20 μL of a concentrated stock solution, prepared as noted above) until the blue color of the radical persisted.

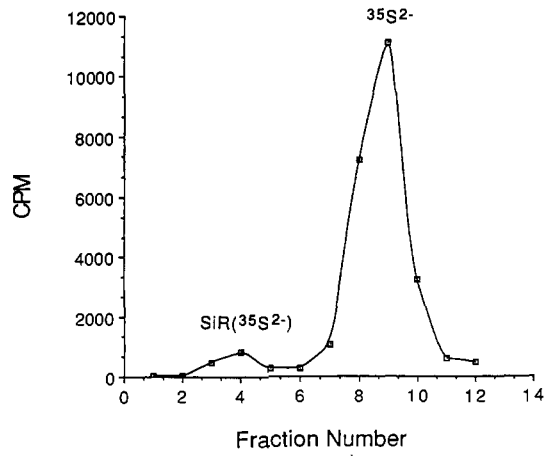


FIGURE 1: Elution profile from a G-100 gel-filtration column following incubation of native oxidized sulfite reductase (28.4 nmol in 0.05 M potassium phosphate buffer, pH 7.6) with 17 equiv of $^{35}S^{2-}$ for 20 min (total volume = 185 μL). The radioactivity of each tube was monitored by scintillation counting (Beckman LS7000).

Reaction of SiR($^{35}S^{2-}$) with SO_3^{2-}. Approximately 20 equiv of SO_3^{2-} was added to 140 μL of a solution containing 20.9 nmol of $^{35}S^{2-}$-labeled sulfite reductase [SiR($^{35}S^{2-}$)] in 50 mM potassium phosphate buffer (pH 7), and the mixture was deoxygenated by purging with oxygen-free nitrogen. Over a period of 1–2 min, 10-μL aliquots of a stock solution of zinc-reduced MeV^{2+} were anaerobically added until the blue color persisted. The reaction mixture was loaded onto the gel-filtration column and eluted with equilibration buffer as noted above.

GC/MS. Experiments were carried out on a Hewlett-Packard GC/MS (HP 5890A GC; HP 5970B MSD). Sulfite reductase was labeled with $^{34}S^{2-}$ as described for SiR($^{35}S^{2-}$). Typically 140 nmol of enzyme in potassium phosphate buffer (50 mM, pH 7.5, 1 mL) was purged with $N_2(g)$ in a septum-stoppered vial. After acidification with 0.1 M H_2SO_4 (0.5 mL), an aliquot (0.2–1 mL) of the gas from the dead space in the vial was injected by use of a gastight syringe. Recommended manufacturer's conditions were used. Relative abundances of peaks were noted and corrected for relative isotopic abundances of sulfur isotopes and interfering peaks from isotopes of residual $O_2(g)$.

Results and Discussion

Incorporation of $^{35}S^{2-}$ into SiR. Sulfite reductase (28.4 nmol) was incubated with a 17-fold excess of $Na_2{}^{35}S$ in 185 μL of 50 mM phosphate buffer pH 7.6 for 20 min and the sample passed through a Sephadex G-100 column. The first peak in the scintillation profile (Figure 1) coincided with the appearance of SiR, followed by free $^{35}S^{2-}$. If the labeled enzyme was concentrated (by using a Centricon, Amicon Ltd.) and rechromatographed, no peak due to free sulfide was observed. This enzyme fraction was fully active. Control experiments with a variety of redox proteins excluded the possibility of sulfide binding to the protein surface or exchange with a core sulfide in the cluster. Specifically, $^{35}S^{2-}$ was incubated with sperm whale myoglobin (pI ~ 8.1) (Blumberg et al., 1971), high-potential iron protein (HiPIP, *C. vinosum*, pI ~ 3.7) (Bartsch, 1971, 1978), cytochrome c_3 (*D. vulgaris*, pI ~ 10.5) (Tan & Cowan, 1990), and a large cytochrome (*D. vulgaris*, pI ~ 8.9) (Tan & Cowan, 1990). Anions that are weakly bound to the protein surface would not be expected

to remain bound during gel-filtration chromatography, and so on the basis of the control experiments described below we exclude this possibility. Sulfide ($^{35}S^{2-}$) did not bind to myoglobin, HiPIP, or cytochrome c_3 but did weakly bind to the large cytochrome, although complexation was inhibited by addition of cyanide. This most likely arises from coordination to the pentacoordinate heme in this enzyme (Tan & Cowan, 1990). The HiPIP experiment precluded the likelihood of exchange with the cluster. Moreover, we are unaware of any examples from model studies of cluster chemistry where core sulfurs have been found to undergo rapid exchange with sulfide in free solution (Reynolds & Holm, 1981). Facile binding to the nonbridging axial site of oxidized siroheme is unlikely since this is already occupied by a strongly interacting ligand (Huynh et al., 1984; Cowan & Sola, 1990), while this site has a low affinity for S^{2-} (Janick & Siegel, 1983). Furthermore, the relatively rapid incorporation of $^{35}S^{2-}$ relative to typical rates of ligand binding for this class of enzyme (Janick & Siegel, 1983) does not support coordination to the nonbridging site. Additional control experiments were performed. First, the number of $^{35}S^{2-}$ species binding to the protein was quantified to one (see below). Over a long time scale (1 h) it was noted that additional $^{35}S^{2-}$ (<1 equiv) was incorporated. This may result from either slow substitution of the nonbridging axial site (Janick & Siegel, 1983) or formation of persulfide after addition to disulfide bonds. Experiments on related proteins have shown that cyanide binds strongly to the axial site but does not displace the bridging ligand (Janick et al., 1983; Janick & Siegel, 1983), while CN^- also inhibits the reactivity of sulfide with disulfide bonds. In the presence of CN^- 1 equiv of $^{35}S^{2-}$ was incorporated, and so binding cannot arise from the side reactions noted above. Furthermore, the *exchange* of sulfide rather than *incorporation of additional* sulfide anion is supported by S^{2-} quantitation. Both the colorimetric assay of Siegel (1965) and mass spectrometric analysis of H_2S evolved after acidification of protein samples demonstrated that the total sulfide content of the enzyme, both before and after treatment with exogenous sulfide ligand, is similar. This clearly points to an exchangeable sulfide. Inasmuch as the axial site is unlikely to bind S^{2-} in the oxidized state, the bridging site is the most probable site for such an exchange reaction.[1] Our argument for exchange of the bridging ligand can be summarized as follows. Under the reaction conditions stated above, the number of enzyme-bound sulfides before and after treatment is similar, as determined by both colorimetric and mass spectrometric analysis, and so the reaction is an *exchange* and not an addition process. On the basis of data from radioassays (with $^{35}S^{2-}$) and GC/MS analysis (with $^{34}S^{2-}$), the number of exchangeable sulfides quantitates to one (see below), even in the presence of CN^-, and so binding to the nonbridging face of the siroheme or addition across a disulfide does not occur. Full catalytic activity is retained after the exchange. In view of these results we attribute the reaction with $^{35}S^{2-}$ to exchange of the bridging ligand and subsequently denote this labeled enzyme as SiR($^{35}S^{2-}$).[2]

Quantitation of Sulfide Binding. The following relationship was derived to quantitate the number of bound $^{35}S^{2-}$ ions:

$$R_m = m/(n - m + 1) \quad (1)$$

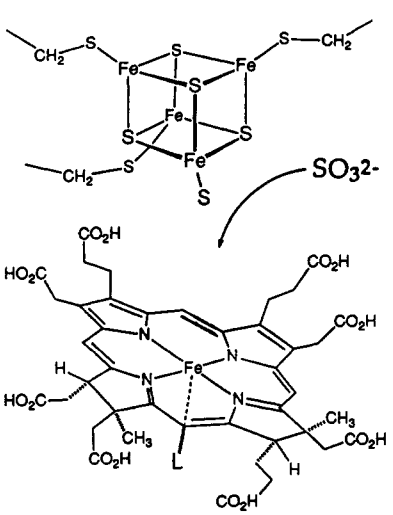
where R_m is the ratio of peak areas {area[SiR($^{35}S^{2-}$)]/area($^{35}S^{2-}$)} in the scintillation profile (Figure 1) for an enzyme possessing m noninteracting exchangeable sites and n is the number of equivalents of $^{35}S^{2-}$ added. The ratio R_m was estimated by cutting around the peaks after plotting the data and weighing the relative masses of each. This was reproducible to within ±20% for the area of each peak when comparing the results from three independent profiles. In the presence of a thiosulfate impurity the expression becomes $R_m = mq/(nq - mq + 1)$, where q is the fraction of sulfide present. One $^{35}S^{2-}$ was incorporated rapidly, independent of the redox state of the enzyme, and full catalytic activity was retained (m varied between 0.85 and 1.2 over a series of three independent experiments). This result was verified by an independent method. Analysis of labile sulfide content from acid-treated SiR($^{34}S^{2-}$) by GC/MS experiments demonstrated the exchange of one sulfide. This was determined from the isotopic ratio of $H_2{}^{32}S$ and $H_2{}^{34}S$, taking into account the enrichment factor of the ^{34}S isotope and the background from isotopes of residual O_2. Incorporation of $^{35}S^{2-}$ was also observed for reduced sulfite reductase. The radioactivity of both oxidized and reduced enzyme after labeling and concentrating were found to be similar within experimental error (±20%), i.e., a similar number of $^{35}S^{2-}$ had been incorporated in both oxidized and reduced samples. Previously it has been shown that the reduced siroheme in *E. coli* sulfite reductase is ca. 10^5-fold more susceptible to binding by ligand anions (Janick et al., 1983). From our studies it is clear that this is not the case for exchange at the bridging site in the *D. vulgaris* enzyme and may be attributed to the distinct coordination mode of that face. The data support exchange of the bridging ligand and suggest that the rate of exchange of this position (both k_{on} and k_{off}) must be rapid, even in the oxidized state. Also, since the binding constant for the bridge must be large, $k_{on} \gg k_{off}$. Although we have no direct evidence for the replacement of the bridging ligand by $^{35}S^{2-}$, all of the data reported in this paper clearly point to that fact. Further evidence for exchange of the bridge ligand derives from the chemistry described below.

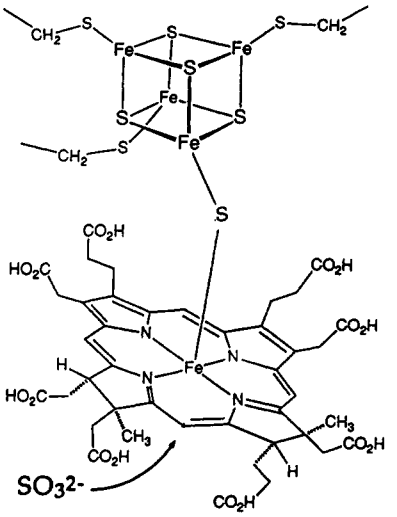
Identification of the Substrate Binding Site: Reaction of SiR($^{35}S^{2-}$) with SO_3^{2-}. The reaction chemistry may result from substrate binding either to the bridging face or to the lower face of the siroheme (Chart II). By careful consideration of viable chemistry we deduced that any reasonable mechanism involving turnover of substrate at the bridging site would lead to displacement of the bridging ligand ($^{35}S^{2-}$). Control experiments were performed; in particular, incubation of oxidized SiR($^{35}S^{2-}$) with an approximately 20-fold excess of SO_3^{2-} in the absence of an electron source resulted in complete retention of radioactivity even after 1 h. Sulfide ($^{35}S^{2-}$) was also retained during turnover (~2 min) with SO_3^{2-} and $MeV^{+\bullet}$ but was slowly exchanged, over a period of 1 h, by product $^{32}S^{2-}$ if the enzyme was left in the reaction solution (Figure 2). Since any chemically reasonable pathway occurring directly between the cluster and siroheme would lead to rapid direct exchange of the bridging ligand, this experiment strongly implicated the chemistry at the lower nonbridging axial site. If the reaction solution was passed through the G-100 column immediately after turnover, only a small amount of radioactivity was lost from the enzyme (Figure 2, top panel). If the solution was allowed to incubate for 10 and 75 min prior to passage through the column, the profiles shown in the

[1] Recently we have obtained evidence that desulfoviridin, a tetrameric dissimilatory sulfite reductase from *D. vulgaris*, is also likely to contain bridging sulfide ligands (unpublished results). This enzyme also possesses exchangeable sulfide; however, the siroheme is pentacoordinate (even after exchange), and so the absence of axial ligand is more readily demonstrated in this case.

[2] Inasmuch as the enzyme derives from a sulfate-reducing bacterium, and so is most likely in contact with exogenous S^{2-} in its natural environment, these results do not appear unreasonable. This may explain the discrepancy with the *E. coli* enzyme.

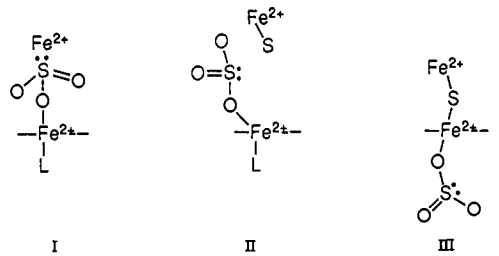
Chart II





middle and bottom panels of Figure 2, respectively, were obtained. Clearly a band corresponding to enzyme-free $^{35}S^{2-}$ is growing in. Our interpretation of these results is that $^{35}S^{2-}$ in the bridge site is not exchanged during enzymatic reduction of SO_3^{2-} to S^{2-}, although subsequent exchange with product S^{2-} (following turnover of SO_3^{2-}) is both possible and expected by analogy with the chemistry described above. This leads to the exchange of bridging $^{35}S^{2-}$ with unlabeled S^{2-} that is free in solution. The implications of this result are discussed later in the paper.

Is Fe–O or Fe–S(N) Coordination Mechanistically Important: Reaction of SiR with NH_2OH and NH_2OMe. Inasmuch as the enzyme catalyzes reductive chemistry, it is reasonable that substrate should bind to a reduced heme center. This is in accord with representative values of the relative rates of substrate binding to oxidized and reduced sirohemes.[3] The question of whether substrate reacts via an Fe–O or Fe–S bound intermediate is also nontrivial. An Fe(II)–O coordination mode may not be the most stable ligation state on thermodynamic grounds; however, it could easily lead to a ferryl–oxo species, which are well-characterized intermediates in a number of oxidoreductase enzymes, and so this route cannot be simply dismissed but deserves serious consideration. For example, intermediates of the following form (only the bridge iron in the Fe_4S_4 cluster has been shown) can be postulated. Complex I is unlikely, on the basis of the results



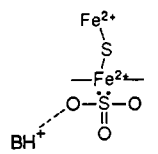
I II III

above. Moreover, the observation that the assimilatory sulfite reductase reduces nitrogenous substrates disfavors such a route since few examples of Fe–S clusters with bound nitrogen ligands are known. After further consideration of potential intermediates (II and III), we disfavor disruption of the bridge in II, while in both cases it is difficult to rationalize how intermediates resulting from cleavage of the S–O bonds might be stabilized.

[3] For SO_3^{2-} binding: k_{on} ~17 s^{-1} (reduced), k_{on} ~5.8 × 10^{-5} s^{-1} (oxidized) (Janick et al., 1983; Janick & Siegel, 1983).

The possibility of reaction via an oxygen-bound species was conveniently tested by using nitrogenous substrates. We noted above that the *D. vulgaris* enzyme will readily catalyze the reduction of NO_2^-, NH_2OH, and NH_2OMe, presumably to NH_3 in comparison to other sulfite- and nitrite-reducing enzymes with analogous prosthetic groups (Siegel et al., 1982; Cline et al., 1986; Crowe et al., 1983a,b). By comparing the reactivities of NH_2OH and NH_2OMe we could readily determine which end of the substrate binds to iron in the siroheme. If Fe–O coordination were important, no turnover would be expected for NH_2OMe since the methoxyl functionality does not readily bind to heme iron. It is reasonable to assume that a common reaction pathway is followed for reduction of oxyanions of both nitrogen and sulfur. We have found that both NH_2OH and NH_2OMe are turned over, with relative rate constants of 10:1 (approximately). No reaction was observed in the absence of enzyme and so substrate is not reduced directly by $MeV^{+\bullet}$ on these time scales. These data argue against a heme–oxygen binding pathway [i.e., reaction proceeds via Fe–N (or S) intermediates]. The bridge linking the chromophores most likely remains intact during turnover to maintain an efficient electron coupling pathway between the cluster and siroheme, ensuring rapid turnover in the two-electron reduction chemistry (McRee et al., 1986).

Proposed Reaction Mechanism. A working model for the reaction pathway is shown in Figure 3. Sulfite binds to the reduced iron center following displacement of the axially bound residue from the siroheme. In common with other π-acid ligands, SO_3^{2-} binds to reduced hemes through π-backbonding from occupied d-orbitals on Fe(II). The pK_a for uptake of H^+ by SO_3^{2-}(aq) is ca. 6.9 (Chemical Rubber Co., 1982), although this may be influenced slightly by coordination to Fe(II). The axial protein ligand can be implicated as a proton source to facilitate S–O bond cleavage (hydroxide is a better leaving group than oxide). Resonance Raman experiments on the



CN^- adduct of the *E. coli* reductase suggest H-bonding to the distal ligand from a neighboring residue (Han et al., 1989). This may be the proton donor during active turnover of this enzyme. The occurrence of an ionizable residue in the vicinity of the substrate binding site is likely to be a general feature

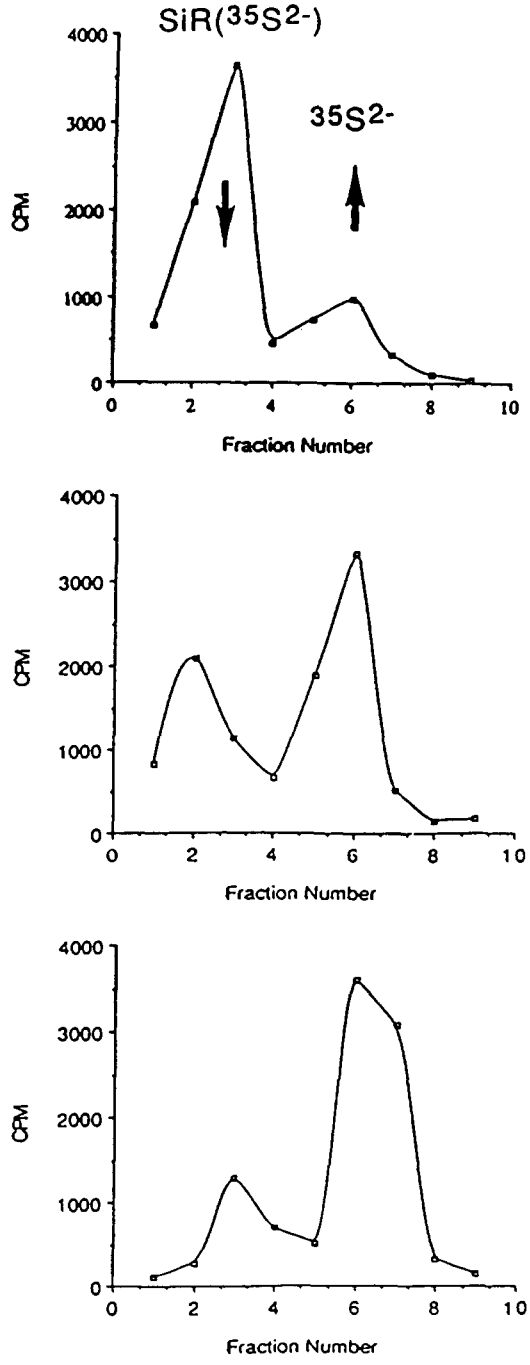


FIGURE 2: Elution profiles monitoring the exchange of enzyme-bound $^{35}S^{2-}$ by product S^{2-} formed during the enzymatic reduction of SO_3^{2-} (20-fold excess) following addition of MeV^+ (25 μL) stock solution. Samples were applied to the G-100 column (top) immediately after turnover, (middle) with 10-min incubation after turnover, and (bottom) with a 75-min incubation after turnover.

in this class of enzyme to facilitate cleavage of S(N)–O bonds. NMR evidence (Cowan & Sola, 1990) suggests displaced histidine as a candidate; however, the same role could be fulfilled by any neighboring residue possessing an ionizable proton. Repetition of the reductive cleavage of the remaining S–O bonds results in bound sulfide that can be displaced by additional substrate or binding of the axial residue. For convenience, changes in the redox state of the siroheme are depicted as occurring at the iron center. The chemistry may, however, proceed via a siroheme π-cation radical since coordination of π-acid ligands may lower the reduction potential of the ring, making ring oxidation more favorable than oxidation of the metal center (Fe^{2+} to Fe^{3+}) (Sullivan & Strauss,

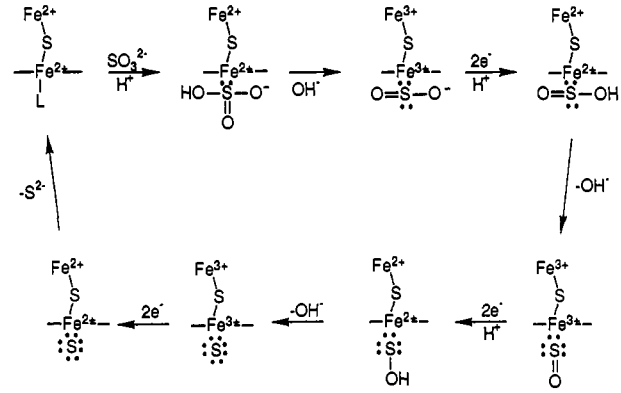


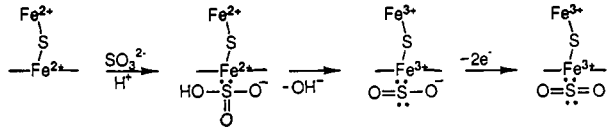
FIGURE 3: Schematic outline of the proposed reaction pathway. Only the bridge iron of the cluster is shown. For convenience of electron counting, oxidation and reduction of the siroheme is depicted at the iron center (see text). Sulfite binds to the reduced iron center following displacement of an axially bound protein residue (L) from the siroheme.

1989; Chang et al., 1981; Fujita & Fajer, 1983). The likelihood of a concerted three-electron reduction process, utilizing the oxidized siroheme ring in addition to oxidized metal centers in the ring and cluster, is small since no chemically stable intermediates can arise from such a pathway.

Binding of SO_2 vs SO_3^{2-}. In neutral or basic solution the equilibrium

$$SO_2 + 2H_2O \rightarrow HSO_3^- + H_3O^+$$

lies to the right (Cotton & Wilkinson, 1988). However, there exists a rich chemistry of SO_2 complexes within the iron group of metals, and so the possibility of a siroheme-bound SO_2 cannot be dismissed. Siegel has previously described how the reaction of sulfite with fully reduced sulfite reductase (*E. coli*) produced, after subsequent two-electron reoxidation, a species that was quite distinct from the complex formed by reaction of sulfite with native fully oxidized enzyme (Janick et al., 1983). The former complex was not characterized.[4] In keeping with the chemistry depicted in Figure 3, this observation is most readily explained by the sequence outlined below.

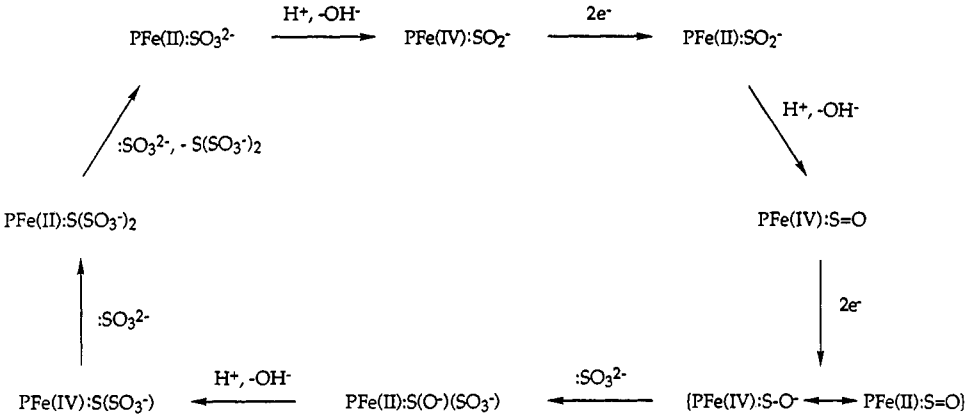


Addition of SO_3^{2-} to reduced enzyme followed by reoxidation with ferricyanide can result in bound SO_2. Recent EPR experiments on model ferric isobacteriochlorins have demonstrated that these complexes can readily bind π-accepting ligands (e.g., CO), even in the oxidized state (Sullivan & Strauss, 1989). The coordination of SO_2 to oxidized native enzyme would therefore not be unexpected. This readily explains the results from Siegel's laboratory, although it should be noted that addition of either SO_2 or SO_3^{2-} to reduced enzyme would produce identical intermediates (above) with identical subsequent chemistry (Figure 3).

Although sulfite reductase activity has also been observed with isolated siroheme, the resulting product is trithionate (Seki

[4] Addition of nitrite to the fully reduced *E. coli* enzyme has also been examined. An EPR spectrum characteristic of a ferroheme–NO complex was reported, although it was uncertain whether this was formed by reaction with reduced enzyme or as a byproduct of the reaction (Janick et al., 1983; Janick & Siegel, 1983).

Scheme I



et al., 1981; Seki & Ishimoto, 1979). A likely pathway for the production of this anion is shown in Scheme I (P = sirohydrochlorin ring). Clearly, several of the intermediates could be rewritten in different resonance forms; however, the general thrust of the mechanism is reasonable. Lone pairs on sulfur are not explicitly shown unless they are involved in bonding, and it is again assumed for purposes of electron counting that all oxidation–reduction chemistry of the siroheme occurs at the iron center. A rationale for the absence of this product following enzymatic assimilatory reduction most likely rests on steric grounds: after an initial reductive cleavage of S–O bonds (cf. Figure 3), additional substrate cannot enter the active site of the enzyme and nucleophilic attack by SO_3^{2-} on siroheme-bound intermediates is prohibited. The nucleophilic chemistry of sulfite is well documented (March, 1985) and the mechanism outlined in Scheme I is analogous to the hydrolysis of an ester, for example, in terms of the "pushing" and "pulling" of electrons. It is also possible to write the reaction chemistry in terms of Fe(II) species throughout, suggesting that the metal simply serves to polarize the Fe–S linkage. In either event the overall scheme is similar: a siroheme-bound sulfoxy intermediate is activated toward nucleophilic attack by sulfite. Although not commonly discussed, the chemistry of simple sulfur–oxygen species has been fairly well characterized (*Gmelin Handbook of Inorganic Chemistry*, 1953, 1980). We note that production of thiosulfate and trithionate has been observed for a few other dissimilatory sulfite reductases (Postgate, 1984) and is probably due to a more accessible active site in those enzymes, at least under the conditions of the in vitro experiment. In keeping with the above scheme, thiosulfate would be formed as (cf. Figure 3)

$$:SO_3^{2-} + PFe(IV)\text{–}S^- \rightarrow PFe(II):S(SO_3^{2-})$$

The interesting proposal of sulfite reduction via sirohydrochlorins in some desulfoviridins (Moura et al., 1988) may represent a distinct mechanistic pathway.

The role of the coupled cluster in enzymatic systems is presumably to provide an efficient source of a second electron and to prevent the buildup of positive charge at the siroheme. A cluster would also offer an efficient sink for additional electrons from natural redox partners since the reorganization energy accompanying oxidation–reduction of Fe–S centers is low. The bridge promotes electronic coupling to the siroheme, allowing efficient delivery of electrons to the oxidized siroheme.

Relevance to Other Active-Site Models. Although the sulfite reductase heme subunit from *E. coli* has been extensively characterized by a number of physical inorganic techniques and low-resolution crystallography, it would be premature to assume that the emerging active site model of the *E. coli* subunit will rigorously hold for all sulfite reductases possessing coupled cluster–siroheme prosthetic centers. There is reason to believe that variations in both siroheme coordination and the chemical nature of bridging ligands could be quite general rather than an exception. We have recently deduced the primary sequence for the assimilatory sulfite reductase in *D. vulgaris* (Tan et al., 1991). Comparison with published data on related systems demonstrates that there is little overall homology between any of the primary sequences for sulfite or nitrite reductases (Ostrowski et al., 1989). In particular, several possibilities exist for the cluster binding site. The model of the reaction pathway that we have described does not depend on whether the siroheme is penta- or hexacoordinate nor on the identity of any bridging ligand. This appears reasonable; the probable function of any bridge is to provide effective electronic coupling to promote electron transfer between the prosthetic centers. A common reaction pathway may therefore be supported by a number of structural models. We emphasize that the mechanism proposed herein is a working model that best explains the data that is currently available from a number of sources. It will serve as a point of reference for the design of experiments to better define, and if necessary update our mechanistic understanding of enzymatic sulfite reduction.

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