# Sulfite Reductase: Active Site Residues are "Noncatalytic". Comparison of Reaction Energetics for Enzyme- and Siroheme-Catalyzed Reduction of Inorganic Substrates†

Aileen Soriano and J. A. Cowan*,‡

*Evans Laboratory of Chemistry*
*The Ohio State University*
*120 West 18th Avenue, Columbus, Ohio 43210*

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Enzymic catalysis of biological transformations is a well-studied phenomenon. A lowering of the activation barrier for key reaction steps can be achieved either by ground-state destabilization of the enzyme−substrate complex or by lowering of the transition-state energy.[1] In cofactor-mediated reactions, the relative contributions of the cofactor and active site protein residues to enzyme catalysis may be evaluated by comparison of kinetic parameters for the cofactor-mediated reaction relative to the enzymatic reaction. Current wisdom dictates that metal-cofactor-mediated chemistry (both redox and nonredox) commands significant involvement from the protein environment. For example, metal ion dependent nuclease enzymes exhibit substrate hydrolysis rates that are considerably greater than those of metal ion activation alone.[2] Also, functional models for redox enzymes typically show turnover rates that are several orders of magnitude lower than those of the enzyme-catalyzed reaction and often require extremes of temperature and pH or addition of other active species to effect turnover at acceptable rates.[3] Recent studies from our laboratory have focused on enzymatic reduction of inorganic substrates by [$Fe_4S_4$]−siroheme containing enzymes belonging to the sulfite reductase family.[4−6] The siroheme apparently serves as the principal catalytic center, while the cluster contributes as an electron trapping and storage unit.[5a] Isolated siroheme can itself catalyze the reduction of inorganic substrates,[7,8] and so comparison of catalytic parameters allows a direct evaluation of the role of active site residues in modulating both ground- and transition-state energies. In this paper we present a detailed evaluation of the kinetic parameters for catalytic reduction of inorganic substrate molecules by isolated siroheme. The results suggest an unusual noncatalytic role for the active site protein residues in this enzyme class. This conclusion may well be generally valid for a wider range of metal-cofactor-mediated enzyme reactions.

Free siroheme was isolated from either the dissimilatory sulfite reductase (DSiR) from the sulfate reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) or the low molecular weight sulfite reductase (SiR) by standard procedures,[8,9] and it was quantitated using the published extinction coefficient ($\epsilon_{595nm} = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$) for the cyanide adduct.[10] Following isolation, the siroheme was demonstrated to be fully metalated by standard analytical procedures.[10] Similar results are obtained irrespective of the source of the siroheme. At pH 6, a plot of initial velocity versus sulfite concentration showed an inverted shape (Figure 1A) that had been observed previously but not explained.[8] We rationalize this plot as an example of uncompetitive substrate inhibition, which is manifest in terms of a reaction mechanism (Scheme 1) that we have previously proposed to explain the high levels of trithionate product obtained from the siroheme-catalyzed reduction of sulfite.[5a] Figure 1A also shows a fit to the data using a standard equation (1) for substrate inhibition,[11] which has been modified to account for the involvement of two $SO_3^{2-}$ species in formation of a trapped trithionate intermediate. At pH ~ 7, substrate inhibition

$$v_0 = \{k_{cat}[\text{siroheme}][SO_3^{2-}]\}/\{K_m + [SO_3^{2-}](1 + [SO_3^{2-}]^2 K_i^{-1})\} \quad (1)$$

is less significant, although $k_{cat}$ and $K_m$ parameters are similar to those obtained at lower pH (Figure 1A). This observation is consistent with a previous report that desulfoviridin-catalyzed sulfite reduction leads to significantly less trithionate by product formation at pH 7 than at pH 6[12] and is consistent with an increase in reactivity of sulfite toward the bound intermediate as a result of protonation of either sulfite or bound thiosulfate at pH levels below 7.[13] Free siroheme was also found to catalyze reduction of $NO_2^-$ and $NH_2OH$ substrates. In contrast to the inverted plot observed for sulfite reduction, the reaction of nitrite and hydroxylamine exhibits standard Michaelis−Menten kinetics (Figure 1B), since neither is sufficiently nucleophilic to react with siroheme-bound intermediates.

Steady-state kinetic parameters can be reasonably considered in terms of a Michaelis−Menten model, where $k_{cat}$ reflects activation energies and $K_m$ reflects ground-state binding energies.[1,5] The magnitude of $\Delta G^*$ can be directly determined at any temperature from eq 2, where $k$, $R$, and $h$ are the Boltzmann, gas, and Planck constants, respectively. Evaluation of the energy

$$k_{cat} = (kT/h)\exp(-\Delta G^*/RT) \quad (2)$$

barriers for turnover of each substrate listed in Table 1 reveals that for free siroheme the $K_m$ and $k_{cat}$ values are generally smaller and larger, respectively, than for the enzyme-bound cofactor.[14]

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(7) Seki, Y.; Ishimoto, M. *J. Biochem. (Tokyo)* **1979**, *86*, 273−6. It is suggested that the preliminary experiments described in the referenced paper demonstrate sulfite reductase activity for metal-free sirohydrochlorin. However, activity was observed only if iron ion was added to the solution, and it disappeared with prior addition of an iron chelator or passage through a chromatographic column. Porphyrinic materials bind iron under these conditions, and so the active species is most likely siroheme. In later work [Seki, Y.; Sogawa, N.; Ishimoto, M. *J. Biochem. (Tokyo)* **1981**, *90*, 1487−92] only fully metalated siroheme was discussed. In our hands, neither free iron ion nor sirohydrochlorin demonstrated catalytic turnover.

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(13) Note the following $pK_a$'s for $H_2SO_3$: $pK_1$ 1.8 and $pK_2$ 6.9.[6a] The protonation state of $HSO_3^-$ most likely influences its reactivity toward siroheme-bound intermediates.

(14) We have previously justified the relationship $K_m \sim K_d$ (refs 5 and the following: Liang, W.; Cowan, J. A. *Inorg. Chem.* **1994**, *33*, 4604−6).

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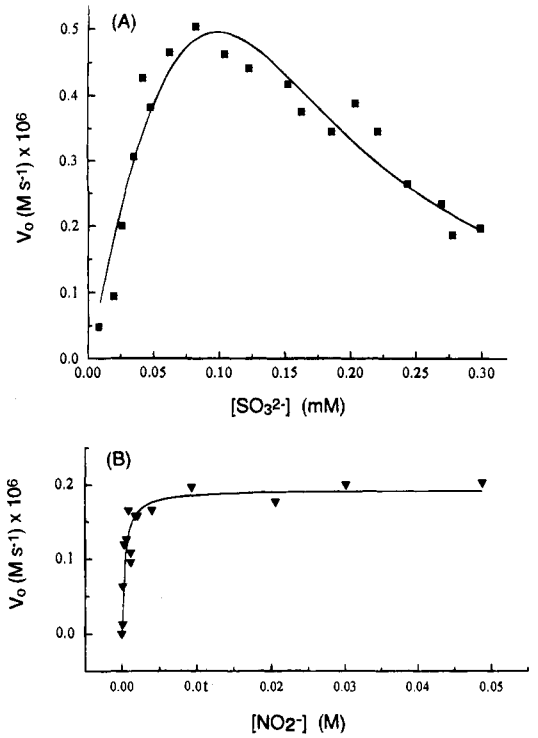
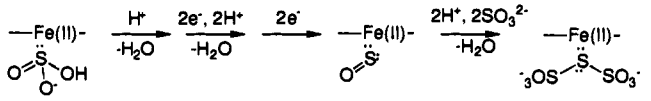



**Figure 1.** Initial velocity plot for turnover of sulfite (A) at pH 6 and nitrite (B) at pH 7. At pH 7, substrate inhibition by $SO_3^{2-}$ is significantly reduced (see text). The data were fitted to eq 1, yielding the following fitted parameters: (A) $V_{max} = 1.4 \times 10^{-6}$ M (mol of siroheme)$^{-1}$ s$^{-1}$, $K_m = 0.1$ mM, and $K_i = 0.02$ $\mu$M for sulfite reduction with [siroheme] = 0.6 $\mu$M (note that the low $K_i$ value indicates a higher affinity of sulfite to the siroheme–intermediate complex than to siroheme alone); and (B) $V_{max} = 1.9 \times 10^{-7}$ M (mol of siroheme)$^{-1}$ s$^{-1}$, $K_m = 0.4$ mM for nitrite reduction with [siroheme] = 0.76 $\mu$M. Experimental conditions are reported in footnote *a* of Table 1.

## Scheme 1




That is, free siroheme is a more efficient catalyst than the enzyme. The only exceptions are catalytic reduction of $NO_2^-$ and $NH_2OH$ by the low-spin sulfite reductase (SiR),[4] these species being 7-fold and 70-fold more active with free siroheme, respectively. However, this is a significantly lower activity enhancement than is observed for normal enzyme catalysis. Significantly, in all cases $SO_3^{2-}$ turnover is mediated by free siroheme at least as well as by the enzymes studied. The unexpected conclusion from these experiments is that the protein matrix apparently does not contribute significantly to catalysis, relative to the siroheme center, but serves simply to afford protection for reduction intermediates from the physiological substrate ($SO_3^{2-}$) and prevent side reactions of the sort illustrated in Scheme 1. Finally, these results suggest that, for other enzymes carrying inorganic redox cofactors, the catalytic role of the protein may be overestimated and formation of a protective reaction pocket may in fact be the dominant function.

**Table 1.** Steady-State Activation Parameters for Siroheme-Catalyzed Substrate Reduction[a]

| substrate | $k_{cat}$ (substr heme$^{-1}$ s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m \times 10^{-3}$ (s$^{-1}$ M$^{-1}$) | $\Delta G^*$ (kcal mol$^{-1}$) | ref |
|---|---|---|---|---|---|
| | | | Siroheme | | |
| $SO_3^{2-}$ | 2.36 | 0.1 | 23.6 | 16.9 | this work |
| $NO_2^-$ | 0.25 | 0.4 | 0.63 | 18.2 | this work |
| $NH_2OH$ | 7.31 | 3.0 | 2.43 | 16.3 | this work |
| | | | DSiR | | |
| $SO_3^{2-}$ | 0.31 | 0.06 | 5.16 | 18.1 | 10 |
| $NO_2^-$ | 0.038 | 0.028 | 1.35 | 19.4 | 10 |
| $NH_2OH$ | 29 | 48 | 0.60 | 15.4 | 10 |
| | | | SiR | | |
| $SO_3^{2-}$ | 0.21 | 0.05 | 4.2 | 18.4 | 15 |
| $NO_2^-$ | 20 | 4.7 | 4.2 | 15.7 | 15 |
| $NH_2OH$ | 2300 | 14 | 164 | 12.9 | 15 |

[a] DSiR is the dissimilatory sulfite reductase enzyme, and SiR is the low molecular weight sulfite reductase enzyme, where each enzyme is isolated from *D. vulgaris* (Hildenborough). Experimental details for steady-state kinetics have been described elsewhere, and similar conditions were used for the experiments described herein.[9,10] Kinetic data were obtained at 298 K at pH 7 by monitoring the decrease in absorbance at 600 nm from the $MeV^{\bullet+}$ radical cation used as an electron source during turnover. A sufficient volume of a previously prepared $MeV^{\bullet+}$ solution was added to give an initial $A_{600} \sim 2.5$ ($\epsilon_{600nm} = 1.3 \times 10^4$ cm$^{-1}$ M$^{-1}$) in a total working volume of 2 mL. The $MeV^{\bullet+}$ stock was prepared by zinc reduction of $MeV^{2+}$: 35 mg in 8 mL of degassed 50 mM phosphate, pH 7.6. Kinetic measurements were made by following the rate of change of the absorbance monitored at 600 nm. The resulting decay profile was analyzed using software from On-Line Instrument Systems. Michaelis–Menten parameters ($k_{cat}$, $K_m$) were determined using commercially available software (Origin) to fit initial velocity plots versus substrate concentration. Reaction conditions were as follows: ($SO_3^{2-}$ turnover) [siroheme] = 0.6 $\mu$M, [$SO_3^{2-}$] = 2–300 $\mu$M; ($NO_2^-$ turnover) [siroheme] = 0.76 $\mu$M, [$NO_2^-$] = 0.1–49 mM; ($NH_2OH$ turnover) [siroheme] = 0.8 $\mu$M, [$NH_2OH$] = 0.1–100 mM. Errors in each measurement are estimated to be on the order of $\pm$0.2 kcal mol$^{-1}$ for $\Delta G^*$.

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