Enzymatic Reduction of Inorganic Anions. Pre-Steady-State Kinetic Analysis of the Dissimilatory Sulfite Reductase (Desulfoviridin) from *Desulfovibrio vulgaris* (Hildenborough). Mechanistic Implications

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Abstract: Pre-steady-state kinetic experiments have been performed on the dissimilatory sulfite reductase (desulfoviridin) from *Desulfovibrio vulgaris* (Hildenborough). Microscopic rate constants for binding (k_2) and reductive cleavage of bonds (k_r) during enzymatic reduction of SO_3^{2-} and NO_2^- have been determined. For NO_2^- reduction the reactivity of reaction intermediates has also been measured and a mechanistic scheme has been devised. The experimental rate constants are as follows: $k_2(SO_3^{2-}) \sim 4.3 \times 10^3\ M^{-1}\ s^{-1}$, $k_r(SO_3^{2-}) \sim 12\ s^{-1}$; $k_2(NO_2^-) \sim 3.6 \times 10^3\ M^{-1}\ s^{-1}$, $k_r(NO_2^-) \sim 14\ s^{-1}$; $k_2(NO) \sim 7 \times 10^5\ M^{-1}\ s^{-1}$, $k_r(NO) \sim 6.5\ s^{-1}$; $k_2(NH_2OH) \sim 24\ M^{-1}\ s^{-1}$, $k_r(NH_2OH) \sim 9\ s^{-1}$. Second-order rate constants for ligand association [$k_2(AsO_2^-) \sim 3 \times 10^3\ M^{-1}\ s^{-1}$; $k_2(HS^-) \sim 1.8\ M^{-1}\ s^{-1}$] are consistent with the dominance of π-acceptor or σ-donor properties, respectively, of substrate molecules. A systematic strategy for the analysis of a multistep enzymatic reduction of an inorganic anion is described.

Introduction

The field of mechanistic inorganic biochemistry remains at an early stage of development. Oxidoreductase reactions of inorganic anions and gaseous molecules are key steps in respiratory and anabolic pathways in cellular metabolism and represent an area of intense investigation.[1-10] Mechanisms for selective binding and activation of inorganic anions or gaseous molecules by metalloenzymes may differ in several important respects relative to common examples of nonredox enzymatic catalysis on organic substrates. The metalloredox prosthetic center both binds and catalytically activates the substrate, while neighboring protein side chains may regulate and optimize the electronic and coordination properties of the prosthetic metal sites toward these functions. Ionizable residues may further contribute to substrate recognition, binding, and activation either by serving as proton donors or providing electrostatic stabilization of charged substrates or intermediates.

With these issues in mind, our laboratory has targeted the [Fe_4S_4]–siroheme-containing sulfite-reducing enzymes ($SO_3^{2-} \rightarrow HS^-$) from *Desulfovibrio vulgaris* (Hildenborough) for detailed study. This enzyme class also catalyzes the six-electron reduction of $NO_2^- \rightarrow NH_3$, although a larger range of prosthetic redox centers and pathways are available for the reduction of nitrite: [Fe_4S_4]–siroheme ($NO_2^- \rightarrow NH_3$),[11] hexaheme ($NO_2^- \rightarrow NH_3$),[7b] copper ion ($NO_2^- \rightarrow N_2, N_2O$).[8] The chemistry of the electron-rich iron isobacteriochlorin (siroheme) has been addressed in model studies.[12-16] Inasmuch as the NO_2^- reduction pathway proceeds via intermediates that can be studied as discrete substrates, this provides a useful probe of the general mechanistic features associated with this class of enzyme. Previous studies

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have focused extensively on structural issues; especially elucidation of the coordination details of the prosthetic redox centers.[8,9] Mechanistic schemes have been proposed only for the copper-containing dissimilatory nitrite-reducing enzymes from denitrifying bacteria, following use of isotopically labeled substrates to elucidate reaction pathways.[8,17] To our knowledge such schemes have not been tested by kinetic studies. In this paper we report microscopic rate constants for ligand-binding and bond-cleavage steps obtained from a pre-steady-state kinetic analysis of the reductive pathway for the desulfoviridin (DV) catalyzed reaction of both SO_3^{2-} and NO_2^- and putative reaction intermediates.

Experimental Methods

Bacterial Growth and Enzyme Purification. *D. vulgaris* (Hildenborough, NCIB 8303) was grown in an enriched Baars medium (ATCC medium No. 1249) as described previously.[19-21] Enzyme isolation and purification also followed normal procedures. Further rigorous purification by FPLC on a Mono Q column (10 × 10 cm) was found to be essential for obtaining spectroscopically pure samples.[19] Enzyme samples were stable indefinitely if stored at −20 °C.

Stopped-Flow Kinetics. A. Preparation of Photoreduced Enzyme. A 5-mL volume of a solution containing 60 μM enzyme, 120 μM deazaflavin, and 15 mM EDTA in 50 mM potassium phosphate buffer (pH 7.6) was deaerated in a 10-mL pear-shaped flask by purging the surface of the stirred solution for 30 min with O_2-free Ar(g). A gas-tight Hamilton syringe was preflushed with Ar(g) and loaded with the Ar(g)-purged solution under positive pressure. The syringe mouth was fitted with a small serum stopper to prevent O_2 diffusion and subsequently immersed in ice–water and irradiated (1000-W lamp, 90% power) for 20 min. During preliminary experiments, reduction was monitored by electronic absorption spectroscopy and irradiation was continued until no further change was observed in the optical spectrum. A second gas-tight syringe was loaded in the same manner with the appropriate substrate concentration (see legends to Table I and Figure 2), without subsequent irradiation. The stopped-flow apparatus was flushed with Ar(g)-purged buffer prior to each experiment.

B. Instrumentation and Methods. Data were obtained with an OLIS (On-Line Instrument Systems, Inc.) stopped-flow apparatus. A broad-band 75-W xenon arc lamp source (Ischio) powered by an OLIS XL150 power supply was filtered through a monochromator (Model H10 by Instruments Sa.) with a resolving power of 8 nm/mm. A photomultiplier tube (Homatsu) usable between 185 and 900 nm was mounted linearly from the source to detect at 438 nm. The piston gas (nitrogen) was delivered at a rate between 9 and 14 mL/s.

Two gas-tight syringes (Hamilton) were loaded with 5 mL each of Ar(g)-purged reactants as described earlier. During the course of the experiment the syringes were occasionally irradiated in situ. Deazaflavin photoreduction of DV maintained the two-electron-reduced enzyme.[18] Reactants were pre-equilibrated at 20 °C prior to mixing. Absorption changes were monitored at appropriate wavelengths (see legend to TableI and Figure 2). Rate constants were determined by use of the OLIS Operating System software (version 12.05) by fitting to proprietary software for rise–fall kinetics.

Results and Discussion

We have initiated a program of experiments to evaluate the detailed molecular mechanism for enzymatic reduction of inorganic substrates. Here we examine the reaction pathway for reduction of substrate molecules possessing N–O and S–O bonds under pre-steady-state conditions and evaluate substrate-binding and bond-cleavage chemistry.

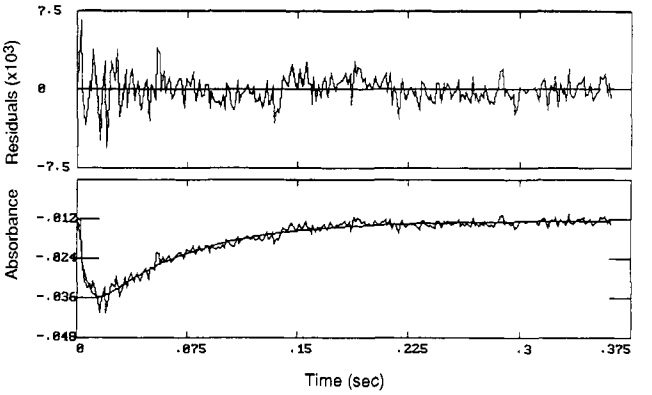

Figure 1. Typical fit to a rise–fall rate profile. The data shown were taken for a final [DV] = 30 μM and [NO_2^-] = 100 mM.

Table I. Pre-Steady-State Kinetic Parameters for Desulfoviridin[a]

substrate/ligand	k_2 (M^{-1} s^{-1})[b]	k_r (s^{-1})
SO_3^{2-}	4.3×10^3 (1′)	12 (2′)
NO_2^-	3.6×10^3 (1)	14 (2)
NO	7×10^5 (3)	6.5 (4)
NH_2OH	24 (5)	9 (6)
AsO_2^-	3×10^3	
HS^-	1.8	

[a] Stopped-flow kinetic data for substrates and ligands were obtained as described in the legend to Figure 1 with the following solution conditions [ligand, λ_{obs} (nm), concentration range (mM)]: NO_2^-, 438 nm, 1–200 mM; NO, 438 nm, 0.1–1 mM; NH_2OH, 438 nm, 1–250 mM; SO_3^{2-}, 438 nm, 10–250 mM; AsO_2^-, 554 and 438 nm, 0.3–10 mM; HS^-, 438 nm, 10–1000 mM. Errors in each measurement are estimated to be on the order of ±50%. [b] Numbers in parentheses after rate constants correspond to the reaction step indicated in Figure 2.

Stopped-Flow Experiments. Absorbance wavelengths used in stopped-flow experiments give rise to prominent changes following redox chemistry or ligand binding, and so both reaction steps were directly monitored (Figure 1, Table I). Relative to the case of oxidized desulfoviridin, there is a pronounced decrease in the absorbance tail from the Soret region (~400 nm) that extends to the Q-bands (~450–630 nm) for reduced enzyme (either with or without exogenous ligand).[22] This affords a useful spectral domain to probe the kinetics of oxidation/reduction and ligand binding by optical methods.

Deazaflavin photoreduction of 60 μM desulfoviridin yielded the two-electron-reduced enzyme.[18] Substrate binding and subsequent reaction to regenerate oxidized siroheme result in absorbance changes that were used to monitor the two-electron reduction of SO_3^{2-} and NO_2^- and intermediates. Excess ligand was reacted with reduced enzyme in the mixing chamber of a stopped-flow instrument under pseudo-first-order conditions. Ligand concentrations are detailed in Table I. For substrates, both the binding (k_2) and reductive (k_r) steps were directly monitored and the resulting optical traces fit by rise–fall kinetic profiles (Figure 1). Second-order rate constants (k_2) were evaluated by variation of ligand/substrate (L) concentration ($k_{obs} = k_2[L]$).

Binding and Reaction Rates. The kinetic parameters detailed in Table I suggest that substrate binding to reduced enzyme is generally rapid and not rate limiting. An exception is found with lower concentrations of NH_2OH. The dependence of on-rates (k_2) on ligand nucleophilicity suggests that binding to the siroheme is associative in character: $k_2(SO_3^{2-}) \sim 4.3 \times 10^3$ M^{-1} s^{-1}, $k_2(NO_2^-) \sim 3.6 \times 10^3$ M^{-1} s^{-1}, $k_2(NO) \sim 7 \times 10^5$ M^{-1} s^{-1}, $k_2(NH_2OH) \sim 24$ M^{-1} s^{-1}. Moreover, if H_2O is bound to the axial site of the "pentacoordinate" high-spin siroheme, its release is not rate limiting. On-rates for AsO_2^- and HS^- [$k_2(AsO_2^-) \sim 3 \times 10^3$ M^{-1} s^{-1}, $k_2(HS^-) \sim 1.8$ M^{-1} s^{-1}] are consistent with the

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(20) Rapid-quench EPR experiments yielded $k_r(SO_3^{2-}) \sim 95$ s^{-1} and $k_r(NO_2^-) \sim 115$ s^{-1} for *E. coli* sulfite reductase.[21a]

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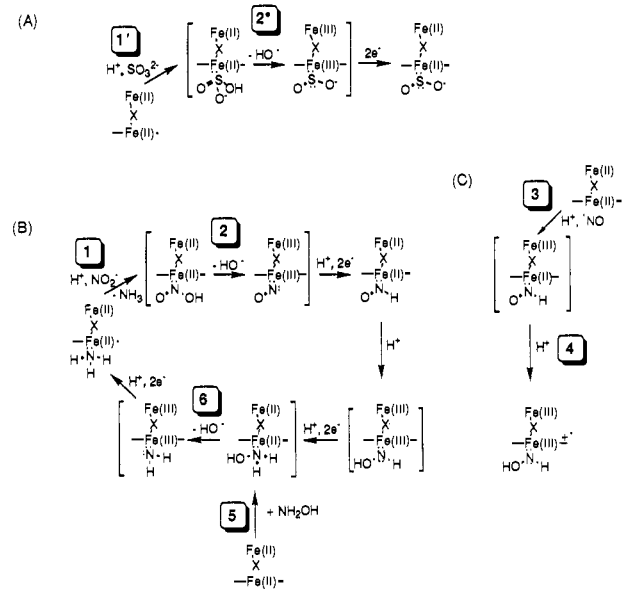


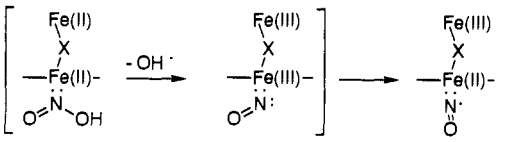
Figure 2. Stopped-flow kinetic data for substrates and ligands obtained as described in Figure 1 and Table I. Only the bridge iron of the cluster is shown. Formal valencies on the iron centers are to aid in electron counting. It is possible that initial oxidation results by electron loss from the ring, rather than the ferrous ion of siroheme. Alternatively, both electrons may initially derive from the metal and the organic chromophore of siroheme. The detailed timing of proton-transfer steps, relative to substrate binding and electron transfer, is not clear at this time. The bridging ligand X is most likely HS^- or S^{2-}.[6] (A) Rate profile for the first step of SO_3^{2-} reduction.[6] (B) Proposed reaction scheme for NO_2^- reduction. (C) Reduction of the nitric oxide radical by the two-electron-reduced enzyme most likely proceeds by a modified pathway (via a radical cation) that is mechanistically consistent with the proposed pathways for NO_2^- and SO_3^{2-} reduction. The numeral adjacent to certain steps on the reaction pathways can be cross-referenced with the rate data in Table I.

dominance of π-acceptor or σ-donor properties, respectively, of substrate molecules. Although AsO_2^- has the ability to both σ-donate and π-accept, the latter appears to dominate.[27] The electron-rich siroheme ring promotes π-backbonding through the d-orbitals of the ferrous ion. It is likely, in fact, that π-backbonding of nitrite and sulfite plays a crucial role in the molecular mechanism of catalysis: promoting tight binding of substrate relative to product, while population of antibonding N–O or S–O orbitals results in a weakening of the bonds that are to be reductively cleaved. The large value of $k_2(NO) \sim 10^5\ M^{-1}\ s^{-1}$ for NO binding is consistent with on-rates determined for other heme proteins [hemoglobin (α/β-subunits) $\sim 3 \times 10^7\ M^{-1}\ s^{-1}$].[23]

Previously we had determined steady-state kinetic parameters for NO_2^- and NH_2OH,[19] and here we extend this to other substrate molecules. With the exception of the case of NH_2OH, reaction rates (k_r) show minimal correlation with steady-state turnover [k_{cat}: $k_r(SO_3^{2-}) \sim 12\ s^{-1}$, $k_{cat}(SO_3^{2-}) \sim 0.3\ s^{-1}$; $k_r(NO_2^-) \sim 14\ s^{-1}$, $k_{cat}(NO_2^-) \sim 0.04\ s^{-1}$; $k_r(NH_2OH) \sim 9\ s^{-1}$, $k_{cat}(NH_2OH) \sim 30\ s^{-1}$],[19] suggesting that bond-cleavage steps are not rate limiting in the early stages of the reaction.[20] For hydroxylamine, $k_r \sim k_{cat}$ and the enzyme saturates when $[NH_2OH] > 250$ mM ($K_m \sim 46$ mM).[19] Both observations are consistent with the second-order binding constant, $k_2 \sim 24\ M^{-1}\ s^{-1}$.

Implications for Enzyme Mechanism and a Molecular Understanding of the Reaction Pathway. In the context of the reaction scheme previously outlined for SO_3^{2-} reduction to HS^-,[6] Figure 2 illustrates the corresponding model for reduction of NO_2^-. That each of the substrates employed (NO_2^-, NO, NH_2OH) reacts readily lends credence to this mechanistic interpretation of the results. We view the reaction as arising through three two-electron

Scheme I



reductive cleavages of N–O (or S–O) bonds.[6] As detailed later, there is a requirement for a number of distinct proton-transfer steps. The reaction of the nitrosyl radical most likely reflects the availability of an additional reducing equivalent in the isobacteriochlorin ring (Figure 2C) rather than turnover of a bona fide intermediate during nitrite reduction.[12] Nevertheless, a transient siroheme-(Fe^{2+}–NO) adduct can be detected by EPR after addition of NO_2^- to reduced DV (characteristic coupling patterns are observed with $^{14}NO_2^-$ and $^{15}NO_2^-$).[21] In related enzymes this evidence has been used to argue for nitric oxide as an obligatory intermediate;[21a] however, this would require only a one-electron reduction. Subsequent reductive steps would require either a further three-electron reduction to produce NH_2OH or a series of one-electron and/or two-electron transfers. Although this cannot be discounted a priori, we find this less appealing inasmuch as it is difficult to rationalize the need for one-electron reductive steps.

Scheme I illustrates an alternative explanation for formation of this nitrosyl complex by invoking back-electron-transfer from "[NO^-]" after the initial reductive addition. No additional electrons are added to push the reaction forward, and so the thermodynamically most stable form of the intermediate is adopted.

Role of Proton Transfer. We have formulated a mechanism that implicitly demands participation by ionizable residues in substrate binding and proton delivery. Clearly, reduction of either SO_3^{2-} or NO_2^- (summarized below, assuming a reaction pH of

$$SO_3^{2-} + 6e^- + 7H^+ \rightarrow HS^- + 3H_2O$$

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O$$

7.6) requires an efficient pathway for transfer of proton equivalents to the active site. In this regard resonance Raman studies of *Escherichia coli* sulfite reductase have suggested hydrogen bond formation to enzyme-bound CN^-.[24] There are, however, two significant differences in the reactions represented by these equations. First, the formal reaction products are formally regarded as an acid (H_2S) or a base (NH_3), respectively. Second, the number of protons that must be delivered to the Fe-bound N or S atom (at pH 7.6) is distinct [one for SO_3^{2-} and three (or four) for NO_2^-]. This is a simple consequence of the inability of nitrogen, unlike sulfur, to accommodate these additional electron lone pairs. We have found the turnover number (k_{cat}) for NO_2^- and NH_2OH to be essentially independent of pH over the range 5–10. This contrasts with steady-state kinetic data for *E. coli* sulfite reductase that demonstrated pH optima of 7.9, 8.6, and 9.5 for SO_3^{2-}, NO_2^-, and NH_2OH, respectively.[26] The dependence of the pH optimum on substrate suggests that for that enzyme the data is not tracking ionization of an amino acid side chain, while the results show no obvious correlation with the pK_a values listed below for these molecules. For desulfoviridin,

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(27) This observation is consistent with the coordination behavior of other potential σ-donor/π-acceptor ligands such as CN^- and CO (unpublished results).

proton transfers are apparently non-rate-limiting. Regular solution pK_a's for substrate and product molecules are as follows: H_2SO_3 (pK_1 1.8, pK_2 6.9); HNO_2 (pK 3.4); NH_3OH^+ (pK 6.0); H_2S (pK_1 12.0, pK_2 7.0); NH_4^+ (pK 9.3).[25] Accordingly, the protonation states of substrate anions and intermediates shown in Figure 2 are appropriate for the pH of 7.6 employed in experiments and simply represent bookkeeping. The precise timing of the proton-transfer steps indicated in Figure 2, relative to substrate binding and electron transfer, remains unclear at this time. For example, does SO_3^{2-} or HSO_3^- bind initially, and is two-electron reductive bond cleavage facilitated by further protonation of the oxygen atom to produce H_2O or is HO^- released? Clearly these points require further evaluation.

In summary, we have described a pre-steady-state analysis of an enzymatic multielectron redox reaction of an inorganic anion and outlined a preliminary mechanstic model that rationalizes these and other published data. The structural and stereoelectronic features of the catalytic apparatus that promote this reaction and the rate-limiting factors in the early stages of sulfite and nitrite reduction are unclear at this time but form the basis for future investigations.

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