Conformational Gating of the Dissimilatory Sulfite Reductase from *Desulfovibrio vulgaris* (Hildenborough). Synthesis, Characterization, and Stopped-Flow Kinetics Studies of 1,5-IAEDANS-Labeled Desulfoviridin†

Siu Man Lui and J. A. Cowan*

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

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ABSTRACT: The siroheme prosthetic center in the dissimilatory sulfite reductase (desulfoviridin) from *Desulfovibrio vulgaris* (Hildenborough) readily binds exogenous ligands in the reduced state, but it does not do so in the oxidized state. In contrast, free oxidized siroheme in solution is observed to bind ligands rapidly. This can only be explained by a structural barrier that precludes ligand binding to the enzyme in the oxidized state but is removed after reduction. These observations suggest a redox-linked structural transformation that provides a gating mechanism for enzyme activation. The rate constants defining these structural perturbations, from oxidized → reduced and reduced → oxidized states, have been determined by monitoring changes in both the natural emission from desulfoviridin and the emission from a surface-bound fluorophore (1,5-IAEDANS). Consistent results were obtained from these two independent experimental measurements (at 25 °C: $k_{ox \rightarrow red} \sim 8\ s^{-1}$, $k_{red \rightarrow ox} \sim 0.05\ s^{-1}$). Activation energies for each transition have been determined from Arrhenius plots (ox → red: ΔG^* 16.5 kcal mol^{-1}, ΔH^* 3.5 kcal mol^{-1}, ΔS^* −43.8 cal K^{-1} mol^{-1}; red → ox: ΔG^* 19.2 kcal mol^{-1}, ΔH^* 11.3 kcal mol^{-1}, ΔS^* −26.6 cal K^{-1} mol^{-1}). These data are used to further develop a functional model previously proposed for this class of enzyme [Lui, S. M., Soriano, A., & Cowan, J. A. (1993) *J. Am. Chem. Soc. 115,* 10483; Lui, S. M., Liang, W., Soriano, A., & Cowan, J. A. (1994) *J. Am. Chem. Soc. 116,* 4531]. The data is consistent with a two-state hypothesis for enzyme activity: an active form (DV^a) that binds and catalyzes substrate reduction, and an inactive form (DV^i) for the resting enzyme. Only the active form of the enzyme need be considered during steady-state turnover. Putative structural mechanisms for conformational gating are proposed. Finally, second-order rate constants have been determined for reduction [at 25 °C: Ti^{3+}, $k_2 \sim 18\ M^{-1}\ s^{-1}$; $MeV^{\bullet+}$, $k_2 \sim 6.5 \times 10^4\ M^{-1}\ s^{-1}$] and oxidation [at 25 °C: $W(CN)_8^{4-}$, $k_2 \sim 200\ M^{-1}\ s^{-1}$] of the siroheme prosthetic center by exogenous redox reagents.

Many electron-transfer proteins, and heme proteins in general, undergo local or global structural changes following oxidation or reduction. Cytochrome *c* and hemoglobin are well-characterized examples (Dickerson & Geis, 1983; Northrup & McCammon, 1984; Takano & Richardson, 1981a,b). Such redox proteins can be said to display the property of conformational gating, albeit in the simplest possible terms. In the case of oxidoreductase enzymes that couple electron-transfer to atom-transfer chemistry, conformational gating may play a more important role in the regulation of enzyme function. Examples of the latter include the α_2 dimeric subunit of nitrogenase, which couples MgATP/MgADP binding to electron transfer from the $[Fe_4S_4]$ ferredoxin cluster (Georgiadis et al., 1992). Moreover, ATP hydrolysis regulates binding of the α_2 dimer (Fe protein) and the $\alpha_2\beta_2$ MoFe protein that contains the catalytic prosthetic center (Kim & Rees, 1992). Cytochrome *c* oxidase also functions by way of a conformationally gated proton-pumping mechanism (Chan & Li, 1990; Larsen et al., 1992; Shapleigh et al., 1992). In this case the structure of the enzyme is dependent on both the oxidation and the coordination states of the component copper and heme redox centers.

Our laboratory is currently investigating the molecular mechanisms of a class of sulfite- or nitrite-reducing enzymes that possess a $[Fe_4S_4]$–siroheme complex as the active redox prosthetic center (Cowan & Sola, 1990; Tan & Cowan, 1991; Lui et al., 1993, 1994; Wolfe et al., 1994). We have focused our studies on two such enzymes from the sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough): a low molecular weight monomeric assimilatory enzyme (Tan et al., 1991) and a larger hexameric dissimilatory enzyme (desulfoviridin, DV)[1] (Lui et al., 1993, 1994; Wolfe et al., 1994). In both cases we have found the enzyme-bound siroheme, the normal coordination site for substrate and other exogenous ligands, to be extremely inert toward ligand binding when the enzyme is in the oxidized state, but it readily binds exogenous ligands in the reduced state. This result is consistent with previous observations made for the oxidized state of the *Escherichia coli* enzyme (Janick et al., 1983). Inasmuch as free siroheme in solution binds exogenous ligand rapidly in either the oxidized or the reduced state (estimated $k_2 \gg 200\ M^{-1}\ s^{-1}$),[2] these results suggest that for most, if not all, enzymes in this class the switch from slow to rapid binding following reduction is gated by a redox-linked conformational change. Note that this need not correspond to a global change in enzyme structure and may be manifest as a local change in the

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[1] Abbreviations: DEAE, diethylaminoethyl; DV, desulfoviridin; FPLC, fast protein liquid chromatography; 1,5-IAEDANS, 5-((((2-iodoacetyl)-amino)ethyl)amino)naphthalenesulfonic acid.

[2] During siroheme extractions from sulfite reductase enzymes, exogenous ligands (CN^- and pyridine) are found to bind to the oxidized heme in fractions of a second. Estimates of second-order rate constants from alkaline solutions of known cyanide concentration place a conservative lower limit of 200 $M^{-1}\ s^{-1}$ on the association rate constant for CN^- binding to free oxidized siroheme in solution.

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orientation of a few amino acid side chains. To determine the rates of these structural transitions, we have surface-labeled desulfoviridin with the fluorescent probe 1,5-IAEDANS, which has been extensively used both as a structural probe and in studies of protein dynamics (Park et al., 1991; Gardner & Matthews, 1991; Kawata & Hamaguchi, 1991; Gorman et al., 1987). In this paper we report results from rapid-stopped-flow kinetics experiments that quantitatively evaluate the rate constants for the structural changes induced by switching from the oxidized to the reduced state (ox → red), and also from the reduced to the oxidized state (red → ox). We demonstrate that these changes are *bona fide* intramolecular pathways and that they do not simply correlate with second-order oxidation or reduction of the prosthetic center. Variable-temperature experiments have been performed to determine the magnitude of the activation barriers for these transformations. By a comparison of these results with data previously obtained from pre-steady-state and steady-state measurements of active site chemistry (Lui et al., 1993, 1994), the structural contributions to the regulation of reaction chemistry have been rationally investigated. The kinetic rate constants and activation barriers evaluated herein are used to further develop the catalytic model described previously. The proposed structural change is consistent with a two-state model for enzyme activity: a transition between an active form (DV^a) that binds and catalyzes substrate reduction and an inactive form (DV^i) for the resting enzyme. Finally, possible structural mechanisms for conformational gating are presented and discussed.

MATERIALS AND METHODS

General Materials and Instruments. Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co.). Sephadex G-25 gel filtration material was obtained from Sigma, and DEAE-52 ion-exchange resin was from Whatman. The fluorophore 1,5-IAEDANS was purchased from Molecular Probes Inc. Titanium(III) chloride was purchased from Aldrich Chemical Co.

Culture Growth/Protein Isolation and Purification. Desulfoviridin was obtained from *Desulfovibrio vulgaris* (Hildenborough, NCIB 8303). Isolation and purification of desulfoviridin followed a modified literature procedure (Wolfe et al., 1994). Final purification was achieved by FPLC (mono Q column, 1 × 10 cm), using two stocks of vacuum-degassed potassium phosphate buffer, pH 7.6 [A, 50 mM; B, 500 mM]. The total running time for FPLC chromatography was 40 min (5 min at 11% B, 17 min at 11 to 100% B, and 10 min at 100% B).

Preparation and Characterization of 1,5-IAEDANS-Labeled Desulfoviridin. Desulfoviridin was labeled with 1,5-IAEDANS by incubating a 1:22 mixture of the enzyme (73.6 μM) and 1,5-IAEDANS (1.6 mM) in the dark for 16 h at 4 °C in 50 mM phosphate buffer (pH 7.5). The solution was then dialyzed against several changes of the same buffer, before gel filtration chromatography on a Sephadex G-25 column equilibrated with the 50 mM phosphate buffer. The protein so obtained was adsorbed onto a DEAE-52 column equilibrated with 50 mM phosphate (pH 7.5) and eluted by a gradient made up of 1 L each of 50 mM phosphate and 350 mM phosphate (pH 7.5). Final purification was achieved by FPLC as described for native enzyme. To avoid photodecomposition of the fluorophore, the surface-labeled protein was protected from light. The average stoichiometry of 1,5-IAEDANS attached to each enzyme was determined by comparing the absorbance at 336 and 630 nm [ε in units of M^{-1} cm^{-1}: desulfoviridin, $\epsilon_{336\ nm} = 8.35 \times 10^4$ and $\epsilon_{630\ nm} = 5.3 \times 10^4$ (Wolfe et al., 1994); 1,5-IAEDANS, $\epsilon_{336\ nm} = 5.7 \times 10^3$ and $\epsilon_{630\ nm} = 0$ (Haugland, 1992)].

Fluorescence Measurements. Steady-state fluorescence measurements were performed using a Perkin-Elmer luminescence spectrometer, Model LS50B. Instrument parameters were as follows: slit width = 7; scan range of the emission = 300–600 nm; excitation wavelength = 336 nm. All fluorescence measurements were conducted at 25 °C using the reagent concentrations described in the captions to Figures 2 and 3. 1,5-IAEDANS displayed an emission band with $\lambda_{max} \sim 480$ nm .

Oxidation and Reduction of Desulfoviridin for Stopped-Flow Kinetics Studies. For studies of redox-linked conformational gating, the ox → red change was brought about by reduction with titanium(III) citrate ($E° = -480$ mV vs NHE at pH 7.5; Strubl, 1938). In control studies with reduced methyl viologen ($MeV^{\bullet+}$), the radical cation was generated by zinc reduction as described elsewhere (Lui et al., 1994). The red → ox change was carried out with $K_4W(CN)_8$ ($E° = +510$ mV vs NHE; Baadsgaard & Treadwell, 1955). Titanium(III) citrate was made by a modified literature procedure (Zehnder et al., 1976). A 7.9-mL volume of buffer solution containing 200 mM sodium citrate and 50 mM potassium phosphate at pH 8.0 was purged for 20 min with O_2-free Ar(g). A 100-μL volume of titanium(III) chloride (in 20–30 wt % of hydrochloric acid) was taken up with an argon-flushed gastight Hamilton syringe and then quickly transferred to the argon-purged buffer solution. The pH of the resulting titanium(III) citrate solution was adjusted to 7.5 by slow dropwise addition of NaOH (from a 2 M stock) with vigorous stirring under an argon atmosphere. The pH-adjusted titanium(III) citrate solution was stored under an argon atmosphere during experiments. Deazaflavin was synthesized by literature methods (Janda & Hemmerich, 1976). Potassium octacyanotungstate was obtained as a gift from Dr. R. L. McCreery (The Ohio State University).

Stopped-Flow 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) with a detection range between 185 and 900 nm was mounted perpendicular to the source, relative to the sample chamber. The piston gas (nitrogen) was delivered at a rate between 9 and 14 mL/s. When rates were monitored through the fluorophore 1,5-IAEDANS, the excitation wavelength was 336 nm, and the fluorescence emission was monitored by use of a visible long-pass colored glass filter with a cut-on wavelength of 420 nm (Oriel Corporation, filter no. 51280). When rates were monitored by following the natural fluorescence of the enzyme, the excitation wavelength was 280 nm, and a long-pass filter with a cut-on wavelength of 299 nm (Oriel Corporation, filter no. 51225) was used.

All solutions were in 50 mM potassium phosphate buffer (pH 7.5). Reactants were preequilibrated at the appropriate temperature in a water bath, and the stopped-flow apparatus was flushed with an Ar(g)-purged buffer prior to mixing. A volume of an enzyme solution was deaerated by purging the surface of the stirred solution for 30 min with O_2-free Ar(g). Two gas-tight syringes (Hamilton) were loaded with 5 mL each of Ar(g)-purged reactants. For studies of the conformational change accompanying the ox → red enzyme

transition, one gastight syringe was loaded with an enzyme solution, while the other was loaded with titanium(III) citrate solution. For studies of the conformational change accompanying the red → ox enzyme transition, one gastight syringe was loaded with a photoreduced enzyme solution, while the other was loaded with air-saturated $K_4W(CN)_8$ solution. To effect deazaflavin photoreduction (Massey & Hemmerich, 1978), a 5-mL volume of a solution containing 15.7 μM enzyme, 120 μM deazaflavin, and 15 mM EDTA in potassium phosphate buffer (50 mM, pH 7.5) was argon-purged for 30 min and loaded into a gastight Hamilton syringe that had been preflushed with Ar(g). The syringe mouth was fitted with a small serum stopper to prevent O_2 diffusion and the entire syringe was subsequently immersed in ice water and irradiated (1000-W lamp, 90% power) for 20 min to promote deazaflavin photoreduction of DV. During the course of the experiment the gastight Hamilton syringe containing the reduced enzyme was irradiated for 2 min to ensure retention of a high fraction of the two-electron-reduced enzyme during the prolonged experiments. A water filter was placed between the syringe and the lamp. Rate constants were determined by use of the OLIS operating system software (version 12.05) by fitting to proprietary software.

RESULTS

Surface Labeling. Desulfoviridin was labeled with 1,5-IAEDANS by stirring an approximate 22-fold excess of the modifying agent with the enzyme for 16 h at 4 °C. The reaction between 1,5-IAEDANS and the enzyme is a simple nucleophilic substitution of the iodo functional group by a cysteine thiol group on the enzyme (Gorman et al., 1987). The stoichiometry of the modification was found to be ~2.4 mol of 1,5-IAEDANS per mole of DV, as determined from the relative absorbances of the enzyme at 336 and 630 nm and the fluorophore at 336 nm. The ratio was significantly lower when the reaction was carried out for shorter periods of time, but no more than 3 equiv of label was taken up by the enzyme, irrespective of reaction time. This is consistent with our previous estimates of surface-accessible cysteines (2–4) (Wolfe et al., 1994) determined by the Ellman reaction (Robyt & White, 1990). The p*I* value for the modified enzyme is similar to that of native (p*I* ~ 4.4), and so the surface-modified enzyme demonstrated elution profiles similar to those of native enzyme during purification by ion-exchange chromatography. The elution profile following the final FPLC purification was similar, irrespective of whether the enzyme peak was monitored by siroheme absorption or 1,5-IAEDANS emission. When the labeled enzyme was stored in 50 mM phosphate at pH 7.5 and −20 °C, its fluorescence characteristics remained unchanged for months.

Characterization of Modified Desulfoviridin. The absorption maxima and extinction coefficients of the surface-labeled enzyme are similar to those of the unmodified enzyme. The steady-state turnover rate (k_{cat} ~ 20 NH_2OH s^{-1} $heme^{-1}$) of the labeled desulfoviridin is similar to that of native enzyme (k_{cat} ~ 29 NH_2OH s^{-1} $heme^{-1}$).

The concentration of enzyme was conveniently measured at 630 nm, where 1,5-IAEDANS shows no absorbance. Figure 1 shows the emission profile for similar concentrations of 1,5-IAEDANS in the free and enzyme-bound forms. The 1,5-IAEDANS emission peak from the modified enzyme at 480 nm is only 2 nm different from the signal from the free fluorescent probe; however, the relative emission intensity of the surface label is approximately 17% that of the fluorescent probe, most likely as a result of energy transfer to siroheme.

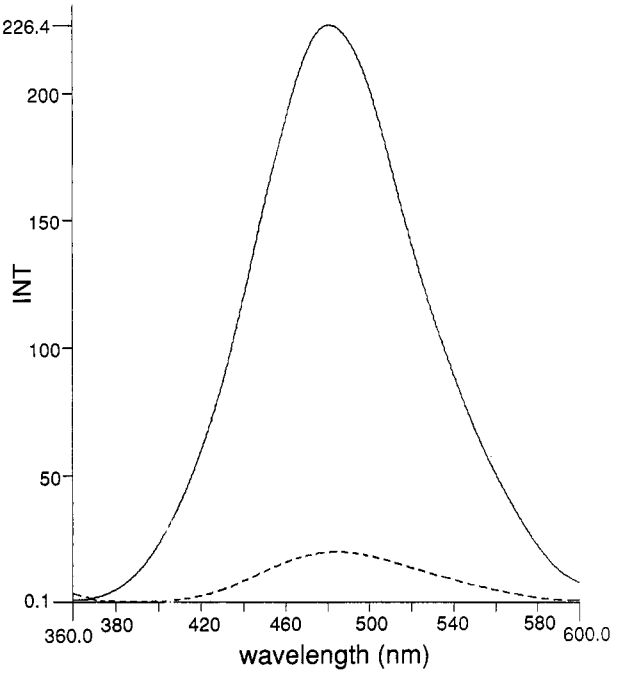

FIGURE 1: Emission profiles for free (—) versus enzyme-bound (- - -) 1,5-IAEDANS. In each case [IAEDANS] ~132 μM. The enzyme concentration for the bound form is ~60 μM.

Note that this fluorescence change is not the feature monitored during gating. Rather, subsequent changes in emission arising from the different environments for 1,5-IAEDANS in the two conformational states give rise to the rate profiles in Figures 2 and 3.

Kinetics Studies. To evaluate the rate of conformational change arising from electron transfer to or from the redox prosthetic centers, we have monitored the change in fluorescence emission both from a fluorophore (1,5-IAEDANS) attached to surface residues (Figure 2) and also from a weak natural emission (presumably from Trp or Tyr residues) (Figure 3). The results obtained with each method are summarized in Table 1 and, as detailed below, were found to be entirely self-consistent. Both the ox → red and red → ox transformations have been examined. Appropriate control experiments to demonstrate that the emission changes observed do not arise through simple oxidation or reduction of the redox chromophores have been carried out. Also, rates were found to be independent of enzyme concentration and do not correspond to intermolecular quenching.

Oxidized → Reduced. A 300-fold excess of titanium citrate was reacted with an argon-purged solution of oxidized labeled desulfoviridin in the mixing chamber of a stopped-flow instrument under pseudo-first-order conditions. The resulting change in emission was directly monitored, and the optical trace was fit by a first-order kinetics profile (Figure 2A) to yield an observed rate constant of ~8 s^{-1}. To eliminate the possibility that the change in emission intensity monitored during the stopped-flow experiment was actually arising from a change in oxidation state at the prosthetic centers, we examined the dependence of the measured rate constant against the concentration of reductant. No dependence was found over a 7.7–17.6 mM range,[3] and so the change in emission (reflected in Figure 2) presumably results from a first-order

[3] The concentration range of reductant that could be usefully employed was limited by too high an absorbance at higher concentrations of titanium(III) and problems arising from partial oxidation of the reduced enzyme at lower concentrations.

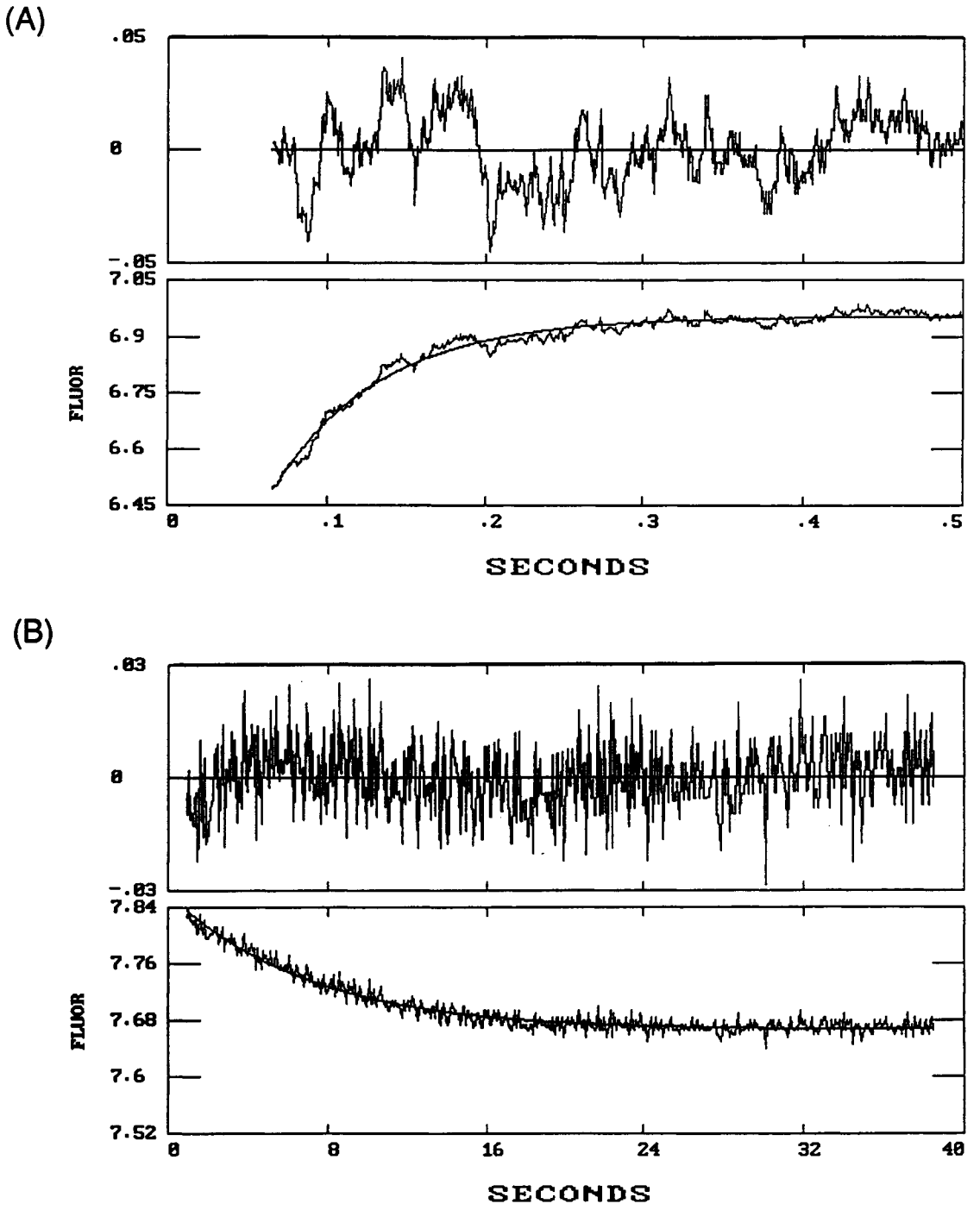

FIGURE 2: Typical fits to a one-exponential rate profile for the change in emission from 1,5-IAEDANS-labeled desulfoviridin. (A) Oxidized → reduced. The data shown was taken for a final [DV] = 35 μM and [Ti^{3+}] = 10.5 mM at a temperature of 36 °C, where $k_{ox \rightarrow red} \sim 13\ s^{-1}$. (B) Reduced → oxidized. The data shown was taken for a final [DV] = 15.7 μM and [$W(CN)_8^{4-}$] = 100 μM at a temperature of 39 °C, where $k_{red \rightarrow ox} \sim 0.14\ s^{-1}$.

structural change following reduction. This conclusion is also supported by further studies on the native enzyme, since desulfoviridin possesses a weak intrinsic fluorescence that could be detected by the photomultiplier tube on our stopped-flow instrument (Figure 3A). The rate of change of the emission intensity for native enzyme was evaluated following reduction of the enzyme in much the same way as previously described for surface-modified desulfoviridin. The first-order rate constant evaluated from these measurements ($k_{red} \sim 8.5\ s^{-1}$ at 25 °C) was in close agreement with the result obtained with the labeled enzyme ($k_{red} \sim 8\ s^{-1}$ at 25 °C). Note also that the relative increase and decrease of emission intensity in Figure 2A or 3A and Figure 2B or 3B, respectively, are entirely self-consistent, with an increase in emission intensity for the reduced enzyme.

As a further control, the rate constant for chemical reduction of desulfoviridin with titanium(III) citrate was independently determined under the conditions employed during the experiment by monitoring the siroheme absorbance change at 438 nm. The observed rate constant was found to vary with the concentration of reductant, and a second-order rate constant, $k_2 = 18\ M^{-1}\ s^{-1}$, was determined at 25 °C (Table 2), while the rate of conformational change during reduction is $8\ s^{-1}$. Clearly the emission from the fluorophore is not directly responsive to the redox state of the prosthetic centers.

Inasmuch as normal steady-state turnover experiments use $MeV^{\bullet+}$ as an electron donor, the second-order rate constant for reduction of siroheme was also evaluated (Table 2) as a

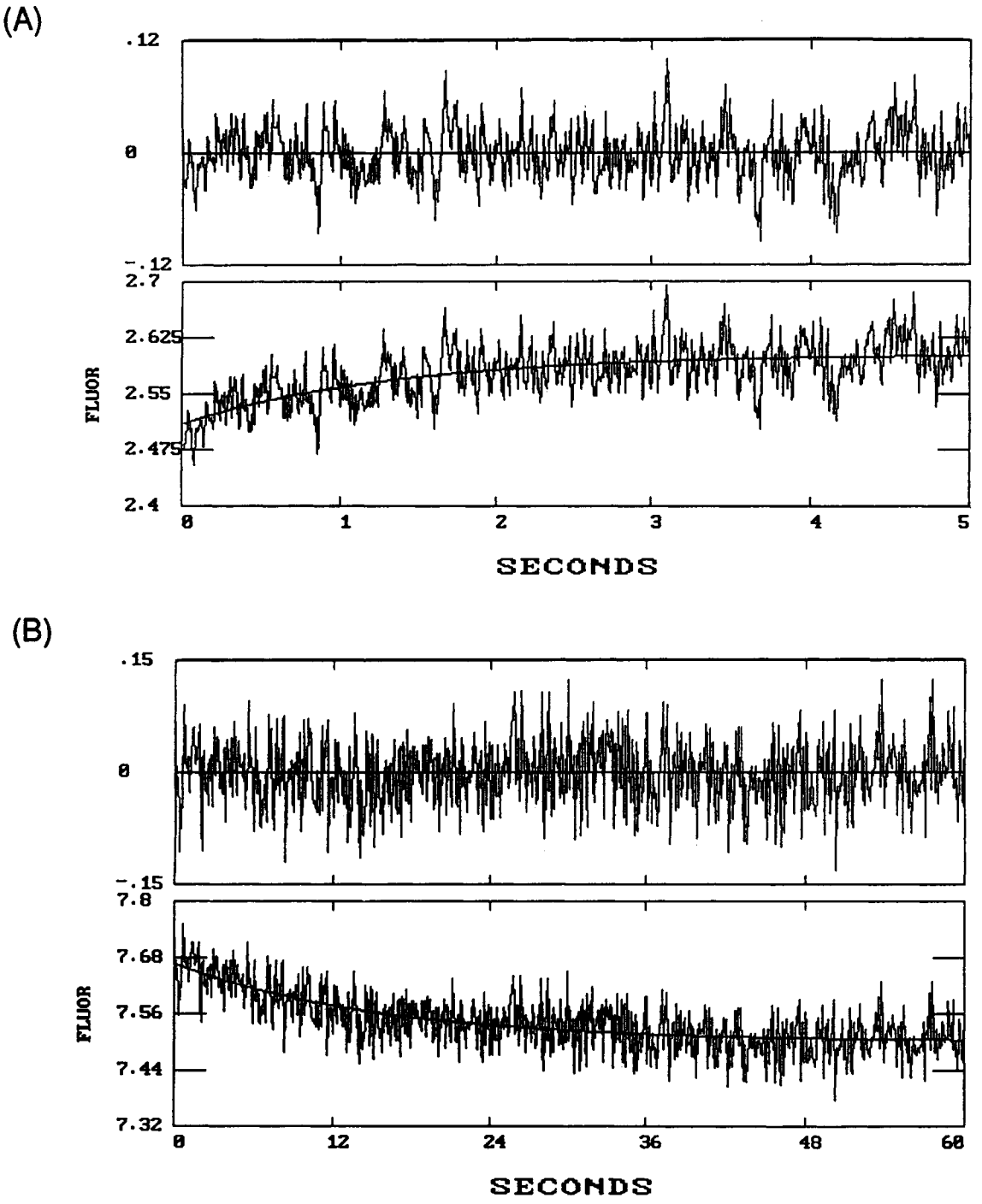

FIGURE 3: Typical fits to a one-exponential rate profile for the change in emission from the natural low-level fluorescence exhibited by desulfoviridin. (A) Oxidized → reduced. The data shown was taken for a final [DV] = 60 μM and [Ti^{3+}] = 11 mM at a temperature of 9 °C, where $k_{ox\rightarrow red} \sim 1.1\ s^{-1}$. (B) Reduced → oxidized. The data shown was taken for a final [DV] = 44 μM and [$W(CN)_8^{4-}$] = 220 μM at a temperature of 25 °C, where $k_{red\rightarrow ox} \sim 0.05\ s^{-1}$.

Table 1: Rate Constants for Redox-Linked Conformational Changes[a]

enzyme	$k_{ox\rightarrow red}$ (s^{-1})	$k_{red\rightarrow ox}$ (s^{-1})
surface-labeled DV	8.0	0.044
unmodified DV	8.5	0.05

[a] Data determined at 25 °C. Rate profiles are shown in Figures 2 and 3. Errors are at least ±50% on all values.

Table 2: Second-Order Rate Constants for Direct Redox Chemistry on the Prosthetic Centers[a]

reductant	$k_{2(ox\rightarrow red)}$ ($M^{-1}\ s^{-1}$)	oxidant	$k_{2(red\rightarrow ox)}$ ($M^{-1}\ s^{-1}$)
titanium citrate	18	$K_4W(CN)_8$	200
$MeV^{\bullet+}$	6.5×10^4		

[a] Errors are at least ±50‰ on all values.

further control. The $k_2 \sim 6.5 \times 10^4\ M^{-1}\ s^{-1}$ determined for $MeV^{\bullet+}$ is more useful for comparative discussions with results from steady-state kinetics experiments made later in the text. Also, these numbers allow the interesting comparison of reduction with a negatively charged complex $[Ti(citrate)_3]^{3-}$, and the positively charged radical cation [$MeV^{\bullet+}$]. Note that methyl viologen could not be used in fluorescence experiments since the radical cation served as an efficient quencher.

Reduced → Oxidized. In a related series of experiments, the rate constant for the conformational change accompanying oxidation of the deazaflavin photoreduced enzyme was

determined. An excess of $K_4W(CN)_8$ oxidant was reacted with deazaflavin photoreduced desulfoviridin in the mixing chamber of a stopped-flow instrument under pseudo-first-order conditions. A first-order decrease in fluorescence intensity was observed (Figure 2B) by monitoring the emission from surface-bound fluorophore. The rate constant $k_{ox} \sim 0.044$ s^{-1} obtained from studies of the labeled enzyme was again confirmed by monitoring the natural fluorescence from native desulfoviridin ($k_{ox} \sim 0.05$ s^{-1}; Figure 3B). In both cases the observed rate constants were independent of the $K_4W(CN)_8$ concentration. Also, in a series of control experiments the rate constant for chemical oxidation of desulfoviridin with $K_4W(CN)_8$ was independently determined under the conditions employed during the experiment by monitoring the siroheme absorbance change at 438 nm. The observed rate constant was found to vary with the concentration of oxidant, and a second-order rate constant $k_2 = 200$ M^{-1} s^{-1} was determined at 25 °C (Table 2). Again, this demonstrates that the emission from the fluorophore is not directly responsive to the redox state of the prosthetic centers.

Variable-Temperature Studies of the Rate of Conformational Change Arising from Reduction or Oxidation of Desulfoviridin. The temperature dependence of the first-order rate constant arising from the structural change following reduction or oxidation of the prosthetic redox centers in desulfoviridin was examined using the Arrhenius rate equation (eq 1 or 2),

$$k_x = (kT/h) \exp -(\Delta G^*/RT) \quad (1)$$

$$R \ln(k_x h/kT) = \Delta S^* - \Delta H^*/T \quad (2)$$

where k, R, and h are the Boltzmann, gas, and Planck constants, respectively. Figure 4 shows the Arrhenius plots obtained from variable-temperature stopped-flow studies of the ox → red and red → ox transitions. The magnitude of the activation free energy (ΔG^*) can be determined directly at any temperature from eq 1. This can be rewritten in the form of eq 2 to determine the enthalpic (ΔH^*) and entropic (ΔS^*) components from the temperature dependence of the rate constant (k). The results from these measurements are given in Table 3, and in each case a substantial entropic component was observed (–43.8 and –26.6 cal K^{-1} mol^{-1}, respectively).

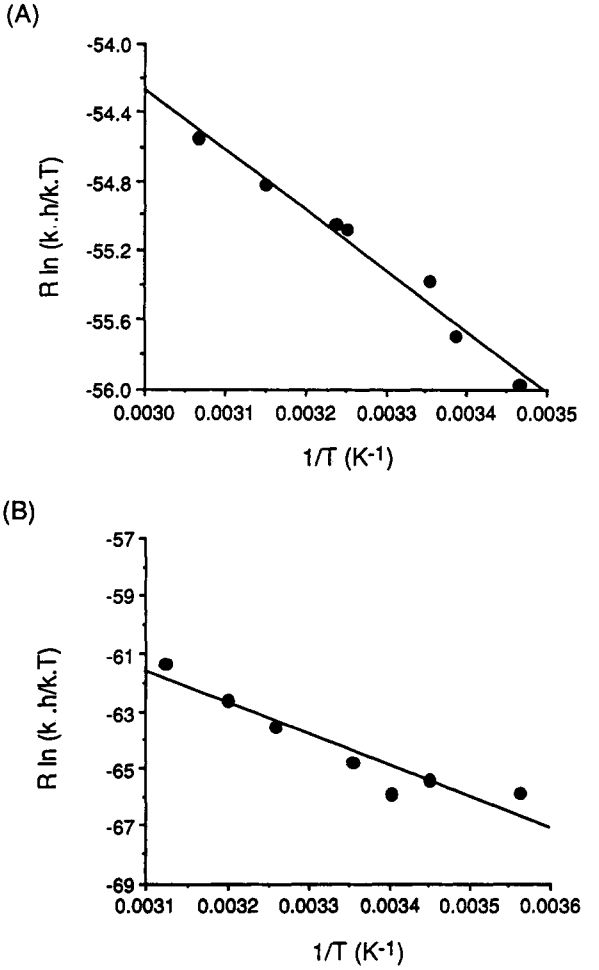

FIGURE 4: Plot of $R \ln(k_x h/kT)$ versus $1/T$ for (A) oxidized → reduced and (B) reduced → oxidized. Enthalpic and entropic components (ΔH^* and ΔS^*) were obtained from the slope and intercept according to eq 2.

Table 3: Activation Energies for Redox-Linked Conformational Changes[a]

transformation	ΔH^* (kcal mol^{-1})	ΔS^* (cal K^{-1} mol^{-1})	ΔG^* (kcal mol^{-1})
oxidized → reduced[b]	3.5	–43.8	16.5
reduced → oxidized[c]	11.3	–26.6	19.2

[a] Errors in each measurement are estimated to be on the order of ±0.5 kcal mol^{-1} for ΔH^*, ±2 cal K^{-1} mol^{-1} for ΔS^*, and ±0.6 kcal mol^{-1} for ΔG^*. [b] Titanium(III) citrate is the reductant. [c] $K_4W(CN)_8$ is the oxidant.

DISCUSSION

Properties of Surface-Bound 1,5-IAEDANS. Desulfoviridin possesses approximately 4 solvent-exposed cysteines, of which we have isolated and purified an enzyme labeled with 2–3 equiv of probe molecules per mole of enzyme. 1,5-IAEDANS-labeled desulfoviridin has been used in stopped-flow experiments to monitor the kinetics of structural changes resulting from reduction and oxidation of the enzyme (Figure 2). Since neither the primary nor the tertiary structure is known, we have not attempted to identify sites of surface binding. However, 1,5-IAEDANS is known to show high selectivity for cysteine residues (Gorman et al., 1987; Andley & Clark, 1988; Lakowicz, 1986), while the results obtained do not depend on the number of surface labels up to the maximum of three noted above. Moreover, since we are measuring a conformational change that is likely to occur with a unique rate constant, we might expect (and observe) a first-order response that is independent of probe position. This stands in contrast to the multiphasic behavior that is commonly observed in *transient lifetime* measurements of excited states in multiply labeled enzymes (Andley & Clark, 1988; Lakowicz, 1986).

Activation Barriers and Reaction Energetics. Previously we have mapped out the energy profiles for reduction of a number of substrate anions and putative reaction intermediates (Lui et al., 1994). Both steady-state and pre-steady-state variable-temperature kinetics experiments were carried out to evaluate the component energies summarized in eq 3. The

$$\Delta G^* = \Delta G_r^* + \Delta G_{of}^* \quad (3)$$

overall activation energy (ΔG^*) for a variety of substrates has been determined from steady-state turnover experiments. The major contributor to ΔG^* is the the free energy of activation for bond cleavage (ΔG_r^*), determined from variable-temperature pre-steady-state experiments, while the term ΔG_{of}^* represents other ill-defined activation barriers. Previously we have shown that the magnitudes of ΔG_{of}^* are typically

small (less than 2.4 kcal mol^{-1}) and ascribed these principally to local (or global) structural changes that might accompany redox chemistry at the prosthetic sites (Lui et al., 1994). To determine whether the ΔG_{of}^{*} contribution could in fact be accounted for by the conformational gating mechanism described above, we evaluated the free energy of activation for both the ox → red and red → ox transformations (Figure 4). Table 3 shows that in each case the activation free energies (16.5 and 19.2 kcal mol^{-1}) were significantly larger than the maximum value of 2.4 kcal mol^{-1} estimated from turnover experiments (Lui et al., 1994). In both cases the barrier we measure has a large entropic component (Table 3), as might be expected for a perturbation of protein structure (Cantor & Schimmel, 1980). We conclude that the proposed structural transitions of desulfoviridin are not observed during normal steady-state turnover, and so the activation barriers determined for these transitions are not manifest in results obtained from previous steady-state experiments (Lui et al., 1994). These results led us to formulate a hypothesis that there might be two conformational states of the enzyme: both active and inactive forms. The rationale for this and important mechanstic implications are developed more fully in the following section.

Conformational Gating and Implications for the Reaction Pathway. We have found desulfoviridin to be remarkably inert toward ligand binding in the fully oxidized state.[4] There appears to be no binding of CN^-, AsO_2^-, or N_3^- to the oxidized siroheme of desulfoviridin, consistent with the rather slow coordination rates observed for cyanide binding to the siroheme of *E. coli* sulfite reductase and spinach nitrite reductase (Janick et al., 1983). In contrast, in free solution siroheme does rapidly bind ligands such as cyanide and pyridine to produce a stable hexacoordinate species.[2] After reduction, we have also found ligand binding to the reduced enzyme prosthetic site to be extremely facile [for example, $k_{on}(SO_3^{2-}) = 4.3 \times 10^3$ M^{-1} s^{-1} and $k_{on}(AsO_2^-) = 3.0 \times 10^3$ M^{-1} s^{-1}] (Lui et al., 1993). We conclude that the kinetic barriers for ligand binding to enzyme-bound siroheme are structural in origin and that ligand binding in the reduced form of the enzyme is promoted by a conformational change that allows easier access of the ligand to the siroheme prosthetic center. Substrate binding is precluded for oxidized enzyme, but access to the active site is possible after reduction. The results and observations reported in the Results section can be understood in terms of the working model shown in Figure 5. Briefly, the enzyme can exist in two structural states: an active form (DV^a) that binds substrate and catalyzes substrate reduction, and an inactive form (DV^i) for the resting enzyme. Only the active form of the enzyme need be considered during steady-state turnover. As noted earlier, these two forms do not necessarily differ markedly in global conformation, and may differ only in the local orientation of a few protein side chains. The distinction at this point is of no relevance for the remainder of the discussion.[5] After reduction, the enzyme undergoes a conformational change to the active form (red-DV^a), at which point it may bind substrate. It is important to note that a structural change is the only reasonable explanation that accounts for the inability of the enzyme-bound siroheme to bind ligands in the oxidized form, while free siroheme in solution does so quite readily.[2] While the bridging ligand and cluster might modulate ligand binding to enzyme-bound siroheme, it should not eliminate binding to the available axial site. The first-order rate constant ($k \sim 8$ s^{-1}) obtained at ambient temperature for the conformational change red-DV^i → red-DV^a resulting from reduction of the redox prosthetic centers is greater than or approximately equal to the turnover rates (k_{cat}) for a range of substrate molecules determined from steady-state kinetics studies. That is, the structural change is not rate-limiting. However, in the section that follows, we will argue that this activation step is unlikely to be involved once steady-state turnover has been achieved.

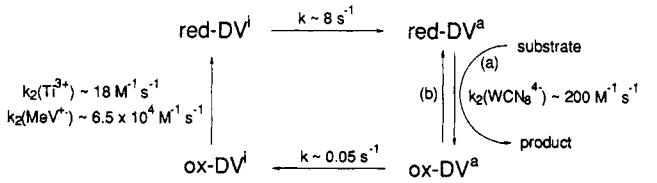

FIGURE 5: Model for redox-linked gating of enzyme activity. The enzyme can exist in inactive (DV^i) and active (DV^a) states. Substrate is precluded from binding to DV^i, but access to the active site is possible after reduction and a conformational transition to the active form, DV^a. The rate constant determined for each of these transformations is noted. During step a, substrates bind to red-DV^a and are reduced to yield product molecules. The rate of oxidation for red-DV^a → ox-DV^a is fairly rapid [$k_2(W(CN)_8^{4-}) \sim 200$ M^{-1} s^{-1}]. Both steady-state and pre-steady-state rate constants have been reported previously for oxidation by reaction with a variety of substrate anions and molecules (Lui et al., 1993). For step b, reduction of the oxidized prosthetic centers by exogenous reductants occurs readily (Table 2), especially for MeV$^{+\cdot}$, the exogenous reductant employed during steady-state turnover. The relatively slow transformation from ox-DV^a → ox-DV^i suggests that the enzyme remains in an active conformation during steady-state turnover.

When bound to the active reduced form of the enzyme, bound substrate undergoes a reductive transformation and the enzyme prosthetic center is oxidized. At this point during normal turnover, the enzyme remains in the active conformation, since the rate constant for the ox-DV^a → ox-DV^i transformation is small (0.05 s^{-1}) in comparison to normal turnover rates. Further reducing equivalents are added from an exogenous electron donor (typically MeV$^{+\cdot}$ in our steady-state turnover experiments). We have previously shown that the steady-state turnover rate is independent of MeV$^{+\cdot}$ concentration (the exogenous electron donor) over a concentration range from 0.05 to 0.25 mM. Consideration of steady-state turnover rates places a lower limit of $k_2 > 2.5 \times 10^3$ M^{-1} s^{-1} on the second-order rate constant for the reverse step (b) in Figure 5, for ox-DV^a → red-DV^a. That is, 2.5×10^3 M^{-1} s^{-1} is the minimum value to support turnover at the observed rates by the exogenous electron donor under the conditions employed in a typical steady-state kinetics experiment.[6] This is higher than the corresponding rate constant ($k_2 \sim 18$ M^{-1} s^{-1}) for the ox-DV^i → red-DV^i transformation determined for reduction by titanium citrate. Since this rate constant is

[4] EPR studies show that the pentacoordinate siroheme in native desulfoviridin remains high-spin (S = 5/2) even after the addition of exogenous ligand. Studies on free siroheme show that the six-coordinate complex is low-spin (S = 1/2) (Kang et al., 1987).

[5] Also, while it is possible that substrate or ligand binding may produce further changes in conformation, there is little direct evidence for this at present. Efforts to measure the rates of such conformational change were unsuccessful; either they are insignificant or they produce a negligibly small change in the fluorescence of the surface label. In any event, given the rate constants determined in this paper and reported elsewhere (Lui et al., 1993), such changes are required to be rapid and not rate-limiting.

[6] Steady-state experiments on typical substrates generally yield a turnover rate (k_{cat}) < 1 s^{-1}, with [MeV$^{+\cdot}$] ~0.2 mM. Taking into account the fact that each enzyme possesses two active centers, an estimate of the rate of electron delivery can be made using the relationship, $k_{cat} = 2(k_2[MeV^{+\cdot}])$. From this, a minimal value of $k_2 > 2.5 \times 10^3$ M^{-1} s^{-1} can be determined. Maximal turnover rates of up to 30 s^{-1} are observed for NH_2OH; however, within error our experimental value of $k_2 \sim 6.5 \times 10^4$ can accommodate this.

insufficient to support the observed turnover rates with $MeV^{\bullet+}$ as electron donor (Lui et al., 1994), we felt it necessary to determine the rate constant for this reductant. Table 2 compares the relative rate constants for reduction with titanium(III) and methyl viologen (18 and 6.5×10^4 M^{-1} s^{-1}, respectively). Assuming similar rate constants for the ox-DV^a → red-DV^a and ox-DV^i → red-DV^i redox reactions (Figure 5), the data for $MeV^{\bullet+}$ satisfies the minimal requirements for steady-state turnover. The difference in magnitudes of the rate constants for titanium citrate and methyl viologen most likely reflects the different electrostatic terms (the enzyme has a p*I* of ~4.4 and will carry a net negative charge at neutral pH) and the differing hydrophobicities of the two reductants, which may allow better penetration by $MeV^{\bullet+}$ at the enzyme surface. This observation is in accord with the Marcus theory of electron transfer (Marcus & Sutin, 1985).

Given the relative rate constants summarized in Figure 5, steady-state turnover apparently must arise from the red-DV^a and ox-DV^a couple. The rate constant for the transformation ox-DV^a → ox-DV^i is small (~ 0.05 s^{-1}) and is not relevant for studies of steady-state turnover. The relatively slow rate of return to the inactive form most likely serves to preserve the enzyme in an active state. The red-DV^i and ox-DV^i forms can only be detected, or implicated, under the fast kinetic conditions employed in these studies and in the absence of substrate molecules. Why the activity of this enzyme should be gated in this way is an intriguing question, but one that will be difficult to address in the absence of a more detailed understanding of the physiological chemistry of the enzyme in vivo.

Putative Structural Mechanisms for Conformational Gating. Protein structural changes resulting from reduction of the prosthetic redox center may result from the influence of siroheme redox state on the pucker of the siroheme ring. Crystallographic studies have clearly demonstrated the flexibility of the reduced isobacteriochlorin ring and the propensity for structural change accompanying redox chemistry at a metalloisobacteriochlorin center (Strauss et al., 1983; Cruse et al., 1982; Barkigia et al., 1982). Such changes of the ring conformation will influence the salt bridges (siroheme carboxylates to positively charged residues) that electrostatically hold the siroheme to the enzyme. Modulation of the electrostatic attraction of the siroheme and protein side chains through these salt-bridge contacts provide an obvious mechanism for communicating a change in redox state of the heme to motion of the enzyme.

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