Enthalpic and Entropic Control of Substrate and Ligand Binding to the Coupled $[Fe_4S_4]$-Siroheme Prosthetic Center of a Dissimilatory Sulfite Reductase

Wen Liang and J. A. Cowan*

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

Received January 6, 1994

Introduction

Ligand binding to metalloporphyrins and derivatives has been extensively investigated in the last two decades.[1,2] This results from the importance of the heme chromophore and derivatives thereof, in a large family of electron-transfer, oxidoreductase, and oxygen-binding proteins and enzymes.[3,4] Linear free-energy relationships have been derived that correlate binding constants with a variety of stereoelectronic parameters.[5,6] Nevertheless, studies on the biological macromolecules themselves and ligand classes related to substrate anions or gaseous species have, for the most part, been limited.[7,8] Two exceptions are the physiologically important oxygen carriers myoglobin and hemoglobin. Both are readily available and are crystallographically characterized.[9,10] As a result, the physicochemical parameters defining binding to these proteins and related model complexes have been evaluated in detail.[11-14] These benchmark measurements provide a frame of reference for any kinetic or thermodynamic studies pertaining to other families of heme proteins.

Elucidation of molecular mechanisms relating to enzymatic redox catalysis is a topic of current importance that requires evaluation and understanding of the kinetic and thermodynamic parameters that underlie substrate binding and activation. Our laboratory is conducting mechanistic investigations on a family of enzymes that catalyze the multielectron reduction of SO_3^{2-}, NO_2^-, and reaction intermediates to H_2S and NH_3, respectively.[15] Such enzymes demonstrate selective binding and activation of substrate anions and neutral species by the $[Fe_4S_4]$-siroheme prosthetic center common to this class of enzyme. In this paper we describe the first detailed study of ligand binding to an enzyme carrying the siroheme chromophore.[8] Thermodynamic parameters for ligand binding to the $[Fe_4S_4]$-siroheme prosthetic center of the dissimilatory sulfite reductase (desulfoviridin) are reported. Since the optical spectrum of desulfoviridin is relatively insensitive to changes in oxidation state or coordination number, binding data have been obtained by kinetic methods.

Experimental Methods

Materials and Methods. Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co). The dissimilatory sulfite reductase (desulfoviridin) was isolated and purified from *Desulfovibrio vulgaris* (Hildenborough, NCIB 8303) by standard protocols.[16]

Steady-State Kinetics and Estimation of Ligand Binding Constants. Procedures for carrying out steady-state kinetics experiments on desulfoviridin have been described elsewhere.[17] Kinetic data were obtained at 298 K by monitoring the decrease in absorbance at 600 nm from the $MeV^{\bullet+}$ radical, which is used as an electron source during turnover. 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.

For inhibition studies, 150 mM hydroxylamine was used as substrate, the enzyme concentration was fixed at 140 nM, and substrate/ligand concentrations were varied as follows: $[SO_3^{2-}]$, 2.5-800 μM; $[NO_2^-]$, 2.5-500 μM; $[NH_2OH]$, 1-500 mM; $[NH_2OMe]$, 10-700 mM; $[CN^-]$, 10-200 μM; $[AsO_2^-]$, 20-400 μM; $[HS^-]$, 20-250 mM; $[N_3^-]$, 20-400 mM. Each inhibitor ligand demonstrated competitive inhibition, and dissociation constants ($K_d = 1/K_a$ = inhibition constant, K_i) were determined by use of eq 1,[18] where all the symbols have their normal meaning.[19]

$$v_0 = \{k_{cat}[\text{enzyme}][NH_2OH]\}/\{K_m(1 + [\text{inhibitor}]/K_d) + [NH_2OH]\} \quad (1)$$

Variable-Temperature Studies. Variable-temperature (VT) steady-state kinetics experiments to monitor the temperature dependence of K_m or K_d were carried out over a temperature range from 278 to 318 K. Enthaplic and entropic components were determined from plots (such as that shown in Figure 1) of ln K_d (=-ln K_a) versus $1/T$ and fitting to eq 2, where all parameters are defined as normal. Typically,

$$\Delta G^\circ = -RT \ln K = \Delta H^\circ - T\Delta S^\circ \quad (2)$$

three to five temperature points were obtained for each substrate or

(1) *Physical Bioinorganic Chemistry*; Gray, H. B., Lever, A. B. P., Eds. Iron Porphyrins Parts I and II; Addison-Wesley: Reading, MA 1983.
(2) (a) Smith, D. W.; Williams, R. J. P. *Struct. Bonding* **1970**, *7*, 1. (b) Traylor, T. G.; Sharma, V. J. *Biochemistry* **1992**, *31*, 2847.
(3) (a) Cowan, J. A. *Inorganic Biochemistry. An Introduction*; VCH: New York, 1993; Chapter 5. (b) Frausto da Silva, J. J. R.; Williams, R. J. P. *The Biological Chemistry of the Elements*; Oxford University Press: Oxford, U.K., 1991. (c) Dawson, J. H.; Sono, M. *Chem. Rev.* **1987**, *87*, 1255.
(4) (a) *Chlorophylls*; Scheer, H., Ed.; CRC Press: Boca Raton, FL, 1991. (b) Weeg-Aerssens, E.; Wu, W.; Ye, R. W.; Tiedje, J. M.; Chang, C. K. *J. Biol. Chem.* **1991**, *266*, 7496. (c) Ellefson, W. L.; Whitman, W. B.; Wolfe, R. S. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3707. (d) *B_{12}*; Dolphin, D., Ed.; Wiley: New York, 1982. (e) Scheidt, W. R.; Lee, Y. J. *Struct. Bonding* **1987**, *64*, 1.
(5) (a) Walker, F. A. *J. Am. Chem. Soc.* **1973**, *95*, 1150. (b) Yamamoto, K. *Inorg. Chim. Acta* **1986**, *113*, 181. (c) Walker, F. A. *J. Am. Chem. Soc.* **1973**, *95*, 1150. (d) Stynes, D. V.; Stynes, H. C.; James, B. R.; Ibers, J. A. *J. Am. Chem. Soc.* **1973**, *95*, 1976. (e) Caughey, W. S.; Fujimoto, W. Y.; Johnson, B. P. *Biochemistry* **1966**, *5*, 3830. (f) Drago, R. A.; Beugelsdijk, T.; Breese, J. A.; Cannady, J. P. *J. Am. Chem. Soc.* **1978**, *100*, 5374.
(6) Cole, S. J.; Curthoys, G. C.; Magnusson, E. A. *J. Am. Chem. Soc.* **1970**, *92*, 2991.
(7) (a) Olson, J. S. *Methods Enzymol.* **1981**, *76*, 631-651. (b) Chan, S. I.; Lee, P. M. *Biochemistry* **1990**, *29*, 1. (c) Blair, D. F.; Witt, S. N.; Chan, S. I. *J. Am. Chem. Soc.* **1985**, *107*, 7389.
(8) Preliminary studies have been reported for the heme subunit of *Escherichia coli* sulfite reductase: Janick, P. A.; Rueger, D. C.; Krueger, R. J.; Barber, M. J.; Siegel, L. M. *Biochemistry* **1983**, *22*, 396-408.
(9) Takano, T. *J. Mol. Biol.* **1977**, *110*, 537-568.
(10) (a) Ladner, R. C.; Heidner, E. J.; Perutz, M. F. *J. Mol. Biol.* **1977**, *114*, 385. (b) Perutz, M. F.; Fermi, G.; Luisi, B.; Shaanan, B.; Liddington, R. C. *Acc. Chem. Res.* **1987**, *20*, 309.
(11) *Hemoglobin and Myoglobin in their Reactions with Ligands*; Antonini, E., Brunori, M., Eds.; Frontiers of Biology Vol. 21; North-Holland Publishing Co.: London, 1971.
(12) Anusiem, A. C.; Beetlestone, J. G.; Irvine, D. H. *J. Chem. Soc. A* **1968**, 960.
(13) Beetlestone, J. G.; Epega, A. A.; Irvine, D. H. *J. Chem. Soc. A* **1968**, 1346.
(14) (a) Parkhurst, L. J. *Annu. Rev. Phys. Chem.* **1979**, *30*, 503. (b) Olson, J. S. *Methods Enzymol.* **1981**, *76*, 652.
(15) (a) Cowan, J. A.; Sola, M. *Inorganic Chemistry* **1990**, *29*, 2176. (b) Tan, J.; Cowan, J. A. *Biochemistry* **1991**, *30*, 8910. (c) Tan, J.; Helms, L.; Swenson, R. P.; Cowan, J. A. *Biochemistry* **1991**, *30*, 9900.
(16) Lui, S. M.; Soriano, A.; Cowan, J. A. *J. Am. Chem. Soc.* **1993**, *115*, 10483.
(17) Lui, S. M.; Liang, W.; Soriano, A.; Cowan, J. A. *J. Am. Chem. Soc.* **1994**, *116*, 4531-4536.
(18) Danenberg, K. D.; Cleland, W. W. *Biochemistry* **1975**, *14*, 28.
(19) Fersht, A. *Enzyme Structure and Mechanism*; Freeman: New York, 1985.

0020-1669/94/1333-4604$04.50/0

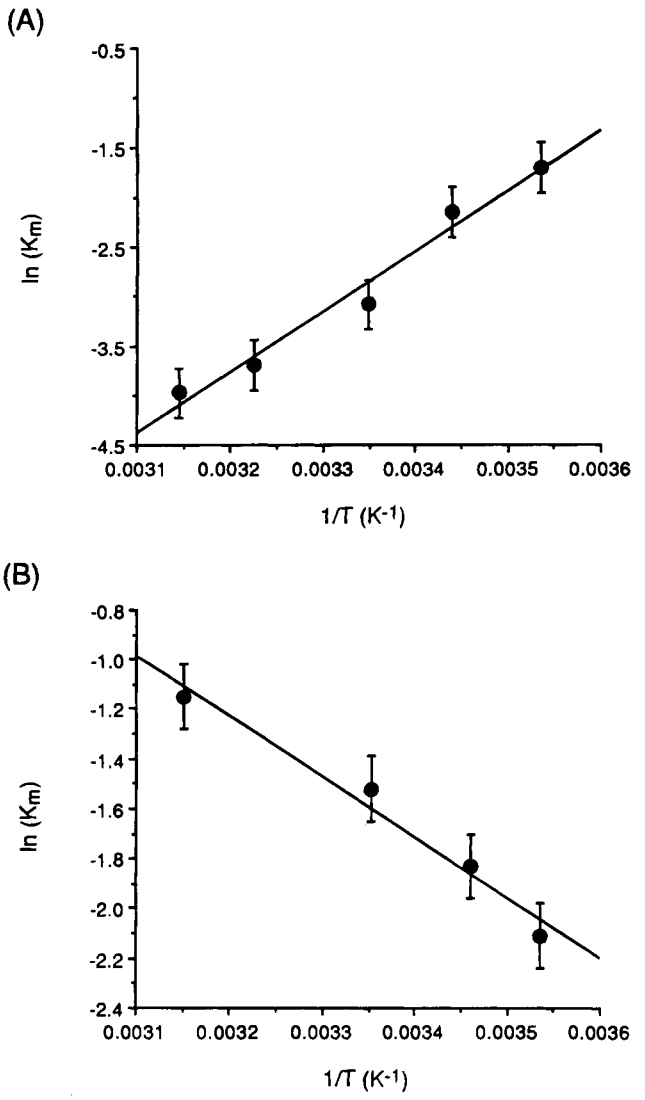

Figure 1. Plot of ln K_m versus $1/T$ for (A) NH_2OH and (B) NH_2OMe. $\Delta H°$ and $\Delta S°$ were obtained from the slope and intercept according to eq 2.

ligand, each point on a VT profile resulting from a complete kinetic analysis at the temperatures selected.

Results and Discussion

Numerous workers have investigated heme–ligand binding equilibria of Hb, Mb, and model compounds in both oxidized and reduced states.[11] More recently, investigations of model complexes were expanded to ring-reduced derivatives (chlorins and bacterio- and isobacteriochlorins).[20-22] While it is often assumed that stability constants can be correlated with heme–ligand bond strengths, previous studies of ligand binding to metalloporphyrins have demonstrated the important role of entropy changes.[6,12] The situation is compounded in studies of ligand binding to metalloproteins since other steric or bonding factors from neighboring protein side chains may be involved. This fact has been reinforced many times by workers who report no apparent correlation between $\Delta H°$ values and ln K (or $\Delta G°$).[6] For example, the data in Table 1 suggest higher binding affinities for π-acceptor ligands, while ligands that bind through σ- or π-donor (for example, N_3^-) mechanisms show lower affinity. However, even for the series of high-affinity π-acceptor ligands (SO_3^{2-}, NO_2^-, AsO_2^-, CN^-), there is no correlation between ln K (or $\Delta G°$) and bond strength ($\Delta H°$). In the case of NO_2^- in particular, binding is apparently entropically driven. The apparent dichotomy can be understood in terms of an isoequilibrium relationship for a series of ligands that bind to a common center. The importance of such isoequilibrium relationships in correlating and understanding thermodynamic data for heme proteins has been highlighted in classic work by Irvine and coworkers on hemoglobin.[12-14] Cole et al. have also discussed this topic in regard to systematic studies with model compounds.[6]

Table 1. Thermodynamic Parameters for Substrate and Ligand Binding to Reduced Desulfoviridin[a]

ligand[b]	K_d or K_m (mM)[c]	$-\Delta G_a°$ (kcal mol^{-1})	$-\Delta H_a°$ (kcal mol^{-1})[d]	$-\Delta S_a°$ (cal K^{-1} mol^{-1})[d]
HSO_3^- (or SO_2)	0.06 ± 0.01	5.8 ± 0.2	7.9 ± 1.8	10.0 ± 2.9
NO_2^-	0.016 ± 0.004	6.5 ± 0.2	−2.2 ± 1.8	−26 ± 7
AsO_2^-	0.9 ± 0.02	4.2 ± 0.3	12.1 ± 1.8	27 ± 4
CN^-	0.26 ± 0.05	4.9 ± 0.2	11.5 ± 2.0	22 ± 3
N_3^-	31 ± 6	2.1 ± 0.2	−7.0 ± 1.8	−25.6 ± 5.4
HS^-	21 ± 4	2.3 ± 0.3	0.1 ± 1.2	−7.3 ± 2.0
NH_2OH	59 ± 12	1.7 ± 0.2	−9.5 ± 1.6	−37.5 ± 2.5
NH_2OMe	192 ± 40	1.0 ± 0.3	5.5 ± 1.5	12.5 ± 1.8

[a] Enthalpic and entropic parameters were determined from a fit to eq 2. Free energies were calculated from equilibrium data (K_d or $K_m = 1/K_a$) at 298 K. Each data set is an average of at least two sets of independent experiments. [b] Ligands are noted in the form most likely to bind to the reduced siroheme, taking into account the ionization constants of each ligand and the binding center. Note that sulfite could conceivably bind in the form of uncharged SO_2. [c] The assumption that $K_d \sim K_m$ for substrates is supported by several lines of argument. These include the similarity of affinities for π-acceptor or σ-donor substrates and ligands. Revised values for K_d would be smaller than the K_m values quoted, while the latter are already at the limit of known binding affinities for siroheme-bound ligands.[8] Also, the resulting data plot (Figure 2) of $\Delta H°$ versus $\Delta S°$ shows a good correlation, which would not be expected if K_m differed substantially from K_d. Other kinetic factors that might make a significant contribution to K_m would be unlikely to show a correlation of $\Delta H°$ and $\Delta S°$ (Figure 2) that matched those exhibited by the non-substrate ligands. [d] The ΔH and ΔS values obtained from VT experiments yield a ΔG value that is within error of the ΔG determined by kinetic studies (column 3).

Analysis of $\Delta H°$ and $\Delta S°$ Components. There can exist a linear relationship between $\Delta H°$ and $\Delta S°$ (eq 3) for a series of

$$\Delta H° = T\Delta S° + \Delta G° \quad (3)$$

related reactions,[6,23] where the ligand, solvent, or both are varied and the natures of the changes induced are similar for the range of iron porphyrins and ligands used. The slope of the resulting line defines an isoequilibrium temperature (T_i), where enthalpy

(20) (a) Halpern, J. *Acc. Chem. Res.* **1982**, *15*, 238. (b) Summers, J. S.; Stolzenberg, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 10559.
(21) (a) Richardson, P. F.; Chang, C. K.; Spaulding, L. D.; Fajer, J. *J. Am. Chem. Soc.* **1979**, *101*, 7736–7738. (b) Chang, C. K.; Fajer, J. *J. Am. Chem. Soc.* **1980**, *102*, 848–851. (c) Barkigia, K. M.; Fajer, J.; Chang, C. K.; Williams, G. J. B. *J. Am. Chem. Soc.* **1982**, *104*, 315–317. (d) Chang, C. K.; Hanson, L. K.; Richardson, P. F.; Young, R.; Fajer, J. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2652–2656.
(22) (a) Stolzenberg, A. M.; Spreer, L. O.; Holm, R. H. *J. Am. Chem. Soc.* **1980**, *102*, 364–370. (b) Stolzenberg, A. M.; Strauss, S. H.; Holm, R. H. *J. Am. Chem. Soc.* **1981**, *103*, 4763. (c) Strauss, S. H.; Holm, R. H. *Inorg. Chem.* **1982**, *21*, 863. (d) Sullivan, E. P.; Grantham, J. D.; Thomas, C. S.; Strauss, S. H. *J. Am. Chem. Soc.* **1991**, *113*, 5264–5270. (e) Melamed, D.; Sullivan, E. P.; Prendergast, K.; Strauss, S. H.; Spiro, T. G. *Inorg. Chem.* **1991**, *30*, 1308–1319. (f) Procyk, A. D.; Bocian, D. *J. Am. Chem. Soc.* **1991**, *113*, 3765–3773. (g) Kang, L.; LeGall, J.; Kowal, A. T.; Johnson, M. K. *J. Inorg. Biochem.* **1987**, *30*, 273–290.
(23) Leffler, J. E.; Grunwald, E. *Rates and Equilibria of Organic Reactions*; Wiley: New York, 1963; pp 324–326.
(24) Wilkins, R. G. *Kinetics and Mechanisms of Reactions of Transition Metal Complexes*; VCH: New York, 1991; pp 96–110.

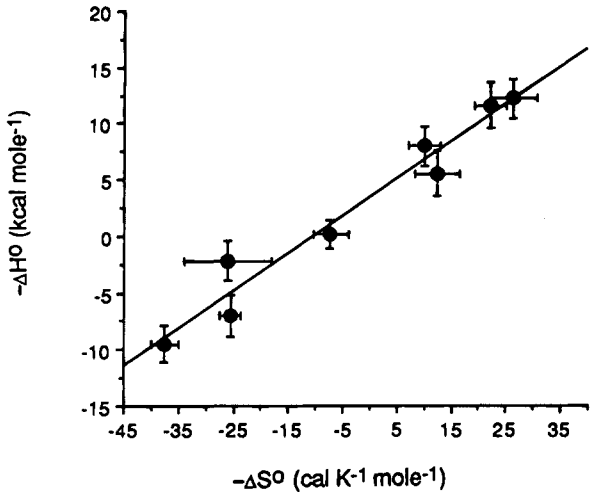

Figure 2. Plot of $-\Delta H°$ versus $-\Delta S°$. The gradient yields an isoequilibrium temperature of 330 K (correlation coefficient of 0.96). Data was taken from Table 1.

and entropy effects cancel and no change is observed in the free energy, but not necessarily in the $\Delta H°$ and $\Delta S°$ components. Although this situation is encountered relatively rarely, the correlation between $\Delta G°$ and $\Delta H°$ will be relatively poor whenever studies are carried out at temperatures close to the isoequilibrium temperature. Figure 2 shows the variation of $\Delta H°$ and $\Delta S°$ for binding of the ligand anions and molecules listed in Table 1. Most of the data lie on a fitted line with a correlation coefficient of 0.96, and the isoequilibrium temperature of 330 K is very close to the value of 298 K used in the determination of $\Delta G°$. At this temperature the variations in $\Delta S°$ values are significant. To account for the different ligand-binding enthalpies determined in studies with hemoglobin, Anusiem et al. have proposed a mechanism involving distinct hydration changes and disruption of specific hydrogen-bonding patterns in the binding pocket for each ligand.[12] Inasmuch as the ligand anions used in our studies may exist in a variety of protonated forms (summarized in Table 1), it is likely that similar considerations may also hold in the case of desulfoviridin; however, the absence of structural data precludes a more definitive evaluation at this time.

Comparison of the data for NH_2OH and NH_2OMe in Table 1 is particularly noteworthy. The methyl derivative of hydroxylamine shows a similar binding constant ($K_m \sim 1/K_a$ increases by less than a factor of 4); however, binding is now controlled by enthalpic rather than entropic terms (Figure 1). The data suggest that the methyl group leads to more favorable interactions with the low-dielectric cavity in the active site pocket, although this is accompanied by restricted motion and a decrease in entropy.

Conclusions. We have systematically evaluated the thermodynamic parameters that define substrate binding and catalysis by the enzyme-bound siroheme chromophore. Future studies will seek to relate the observed differences in thermodynamic binding parameters for heme and siroheme with their functional chemistry.[16]

Acknowledgment. This work was supported by the NSF, Grant No. CHE-8921468. J.A.C. is a Fellow of the Alfred P. Sloan Foundation, a Camille Dreyfus Teacher-Scholar, and a National Science Foundation Young Investigator.

Supplementary Material Available: Plots of all ln K_d versus $1/T$ data (6 pages). Ordering information is given on any current masthead page.