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Electron Paramagnetic Resonance and Optical Evidence for Interaction between Siroheme and Fe_4S_4 Prosthetic Groups in Complexes of *Escherichia coli* Sulfite Reductase Hemoprotein with Added Ligands†

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ABSTRACT: Janick & Siegel [Janick, P. A., & Siegel, L. M. (1982) *Biochemistry 21*, 3538-3547] showed that the EPR spectrum of the reduced Fe_4S_4 center ($S = {}^1/_2$) in fully reduced native ("unligated") *Escherichia coli* NADPH-sulfite reductase hemoprotein subunit (SiR-HP) is perturbed by interaction with paramagnetic ferrous siroheme ($S = 1$ or 2) to yield several novel sets of EPR signals: one set with all g values between 2.0 and 2.8, termed "$S = {}^1/_2$" type, and two sets with the lowest field g value between 4.7 and 5.4, termed "$S = {}^3/_2$" type. The present study has shown that EPR spectra of fully reduced SiR-HP are nearly quantitatively converted to the classical "$g = 1.94$" type typical of $S = {}^1/_2$ Fe_4S_4 clusters when the heme has been ligated by strong field ligands such as CO, CN^-, S^{2-}, and AsO_2^-, converting the ferroheme to $S = 0$. However, the exact line shapes and g values of the $g = 1.94$ differ markedly when different ligands are bound to the heme. Also, optical difference spectra taken between enzyme species in which the heme is kept in the same (Fe^{2+}) oxidation state while the Fe_4S_4 center is reduced or oxidized show that the optical spectrum of the ligated siroheme is sensitive to the oxidation state of the Fe_4S_4 cluster. These results indicate that the heme-Fe_4S_4 interaction of native SiR-HP persists even when the heme Fe is bound to exogenous ligands. We have also found that the g values of the exchange-coupled $S = {}^1/_2$ and $S = {}^3/_2$ type signals of native reduced SiR-HP can be significantly shifted by addition of potential weak field heme ligands—halides and formate—or low concentrations of certain chaotropic agents—guanidinium salts and dimethyl sulfoxide—to the fully reduced enzyme. Such agents can also promote interconversion of the $S = {}^1/_2$ and $S = {}^3/_2$ type signals. These effects are reversed on removal of the agent. Treatment of reduced SiR-HP with relatively large concentrations of chaotropes, e.g., 60% dimethyl sulfoxide or 2 or 3 M urea, leads to abolition of the $S = {}^1/_2$ and $S = {}^3/_2$ EPR signals and their replacement by signals of the $g = 1.94$ type.

Multielectron reduction reactions involve some of the most important and yet least understood enzymatic processes known. Two of these reactions, catalyzed by cytochrome *c* oxidase ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$) and nitrogenase ($N_2 + 8H^+ + 6e^- \rightarrow 2NH_4^+$), respectively, involve enzymes with multiple subunits and multiple prosthetic groups. The complexity of these enzymes has slowed progress in the analysis of the multielectron reduction processes catalyzed by them. In contrast, two other multielectron reduction reactions, sulfite reduction to sulfide ($SO_3^{2-} + 8H^+ + 6e^- \rightarrow H_2S + 3H_2O$) and nitrite reduction to ammonia ($NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$), can be catalyzed by monomeric enzymes which contain only two prosthetic groups—an Fe_4S_4 center and a novel heme, termed "siroheme"—on a single polypeptide chain of M_r ~60 000 (Lancaster et al., 1979; Siegel et al., 1982; Krueger & Siegel, 1982a). Although sulfite and nitrite reductases are physiologically distinct proteins within a given organism (Krueger & Siegel, 1982a), each enzyme is capable of catalyzing both types of multielectron reduction reaction.

The NADPH-sulfite reductase of *Escherichia coli* consists of an oligomeric complex between four "hemoprotein" subunits of M_r 54 600, each of which contains 1 mol of siroheme and one Fe_4S_4 center, and an octameric "flavoprotein" which catalyzes electron transfer from NADPH, the physiological reductant, to the hemoprotein subunits, which serve as the sites of sulfite reduction (Siegel & Davis, 1974; Siegel et al., 1982). The complex can be dissociated in 4 M urea and the monomeric hemoprotein subunit (SiR-HP)[1] isolated free of the flavoprotein by DEAE-cellulose chromatography (Siegel & Davis, 1974). The SiR-HP subunit catalyzes both sulfite and nitrite reduction at high rates if supplied with a suitable artificial electron donor, such as reduced methylviologen (Siegel et al., 1982).

Christner et al. (1981) have shown by Mössbauer spectroscopy that the iron atoms of the heme and Fe_4S_4 prosthetic groups in SiR-HP are exchange coupled, i.e., they must be chemically linked by a bridging ligand in the oxidized enzyme as isolated. Janick & Siegel (1982) demonstrated that SiR-HP

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[1] Abbreviations: Dfl, 5'-deazaflavin; Fe_4S_4, tetranuclear iron-sulfur center; MV^+, methylviologen cation radical; SiR, sulfite reductase; SiR-HP, hemoprotein subunit of *E. coli* NADPH-sulfite reductase hemoflavoprotein complex; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride.

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takes up electrons successively in the heme and Fe_4S_4 centers. EPR and optical spectra of SiR-HP in various oxidation states clearly showed that coupling between the heme and Fe_4S_4 centers is maintained as SiR-HP becomes reduced. Particularly striking is a novel set of EPR signals found in fully reduced SiR-HP by Janick & Siegel (1982), with the majority species seen at $g = 2.53$, 2.29, and 2.07 ("$S = {}^1/_2$" type signal), and minority species seen with g values like those of an "$S = {}^3/_2$" type ground-state species. These signals are presumed to derive from exchange coupling between spins of paramagnetic ferroheme ($S = 1$ or 2) and reduced ferredoxin-type Fe_4S_4 center ($S = {}^1/_2$).

SiR-HP can bind a number of exogenous ligands, including substrates and inhibitory agents, such as CN^- and CO (Siegel et al., 1982). In all cases seen to date, ligand binding appears to involve the heme prosthetic group. With SO_3^{2-} and CN^-, only 1 mol of ligand was bound per mol of SiR-HP, and binding of these agents, as well as CO, was found to be mutually exclusive. Siegel et al. (1982) found that fully reduced SiR-HP, when complexed to CN^- or CO, both strong field heme ligands which can be expected to produce $S = 0$ ferroheme, yielded typical "$g = 1.94$" type EPR spectra characteristic of magnetically isolated reduced Fe_4S_4 centers. [$g = 1.94$ type spectra were also seen in SiR-HP fully reduced in the presence of the perturbant 80% (v/v) Me_2SO.]

Since ligated states of the heme are involved in the multielectron reduction reactions catalyzed by SiR-HP (Rueger & Siegel, 1976), it is important to determine whether or not the coupling between the heme and Fe_4S_4 centers, so evident in the "free" enzyme, is maintained in ligated forms of SiR-HP. It is also of interest to examine the effect of agents which might be able to perturb the protein structure about the SiR-HP active center on the interaction between the two enzyme prosthetic groups. In the present study we have investigated EPR and optical spectroscopic properties of SiR-HP (both reduced and oxidized) in the presence of a number of exogenous agents, including known and potential heme ligands and chaotropic agents. The results show that heme–Fe_4S_4 interaction is maintained on ligation of the heme by a number of compounds. Evidence is also presented which shows that potential weak field heme ligands can promote interconversion of the $S = {}^1/_2$ and $S = {}^3/_2$ type EPR signals characteristic of the exchange coupled heme–Fe_4S_4 center in fully reduced SiR-HP.

Experimental Procedures

The "standard buffer" used throughout the present work was 0.1 M potassium phosphate–0.1 mM EDTA, pH 7.7. *E. coli* NADPH–SiR hemoflavoprotein complex was purified by the procedure of Siegel et al. (1973), and its hemoprotein subunit (SiR-HP) was dissociated and isolated as described by Siegel & Davis (1974) except that 4 M urea was used to minimize loss of prosthetic groups and the isolated protein was extensively dialyzed vs. standard buffer to remove the urea. All chemicals used were reagent grade and were not further purified.

Assays of MV^+–SO_3^{2-} reductase activity were performed as described by Krueger & Siegel (1982a). Photoreduced enzyme samples were added anaerobically to cuvettes containing buffer and MV^+ (reduced with H_2/Pt asbestos) and the oxidation of MV^+ followed spectrophotometrically at 600 nm.

Anaerobic photoreduction of SiR-HP solutions in EPR tubes or optical cuvettes was achieved by the method of Massey & Hemmerich (1978); enzyme solutions contained 10 mM EDTA and Dfl/SiR-HP ratios of 0.2–0.35. Dfl was a generous gift of Dr. D. Seybert. For details of the photoreduction procedure, the anerobic techniques employed, the methods for recording optical and EPR spectra of enzyme samples during the photoreduction process, and the procedures for determining the number of electrons in a particular photoreduced enzyme sample by titration with $K_3Fe(CN)_6$, see Janick & Siegel (1982).

Optical spectra were recorded at room temperature with an Aminco DW-2 dual-beam spectrophotometer. EPR spectra were recorded at X band with a Varian E-9 spectrometer equipped with an Air Products liquid He cryostat. Modulation frequency was 100 kHz and modulation amplitude 10 G in all experiments. Spin concentrations were determined from spectra recorded under nonsaturating conditions either by double integration or by integration of isolated absorption-type peaks by the method of Aasa & Vanngaard (1975). CuEDTA served as standard. The following temperatures and microwave powers were used for quantitation of the various EPR signals in this paper: high-spin ferriheme and $S = {}^1/_2$ type species, 20 K, 50 mW; low-spin ferriheme and $g = 1.94$ type species, 20 K, 10 mW; $S = {}^3/_2$ type species, 8 K, 100 mW (unless otherwise indicated). A Hewlett-Packard 9825A computer equipped with 9874A digitizer and 7225A graphics plotter was used for integrations and simulations of EPR spectra [using the program of Lowe (1978)] as well as for calculation of EPR and optical difference spectra.

Results

Table I shows g values and the spin intensities for EPR signals seen in SiR-HP photoreduced with Dfl/EDTA in the presence of a number of agents. The observed signals have been divided into three classes: (1) the classical $g = 1.94$ type of signal characteristic of magnetically isolated reduced Fe_4S_4 centers; (2) an $S = {}^1/_2$ type of signal with all three g values in the range 2.0–2.8; (3) an $S = {}^3/_2$ type of signal with its lowest field g value in the range 4.7–5.4. Inspection of Table I shows that the particular g values found for an EPR signal of any of the three classes in a sample of reduced SiR-HP depends on the type of potential heme ligand or other agent present. Moreover, the distribution of spins between the three classes of EPR signal in a reduced enzyme sample also varies with the type of agent present. Thus, the $g = 1.94$ type of signal comprised a majority (up to 1.0 spin/SiR-HP) of the observed spins in SiR-HP photoreduced in the presence of CN^-, S^{2-}, CO, AsO_2^-, 60% (v/v) dimethyl sulfoxide, and 2 M urea. The $S = {}^1/_2$ or $S = {}^3/_2$ types of signal, taken together, comprised a majority of the observed spins in HP reduced in the presence of Cl^-, F^-, or Br^- (all at 5 mM), formate (100 mM), 0.2 M guanidinium salts, 40% (v/v) or less dimethyl sulfoxide, and 1 M urea, as well as in enzyme reduced in standard buffer alone.

Figure 1 shows that if one examines the EPR spectra of reduced SiR-HP samples which contain only small amounts of the $g = 1.94$ type of signal (0.11 spin/SiR-HP or less), one finds an inverse relationship between the amount of $S = {}^3/_2$ and $S = {}^1/_2$ type signals seen in the presence of a given agent. The maximum amount of $S = {}^3/_2$ signal expected in fully reduced SiR-HP would be 0.93 spin/mol by extrapolation of the data of Figure 1 to zero spins of $S = {}^1/_2$ species. Since all of the samples used in Figure 1 contained 0.03–0.11 spin/SiR-HP of the $g = 1.94$ type of EPR signal, it is evident that all of the reduced enzyme is accounted for in this extrapolation. (On the other hand, extrapolation of the maximum amount of $S = {}^1/_2$ species to zero spins of $S = {}^3/_2$ species yields only 0.75 spin/SiR-HP. This result suggests that there is a systematic underestimation, as yet unexplained, of the

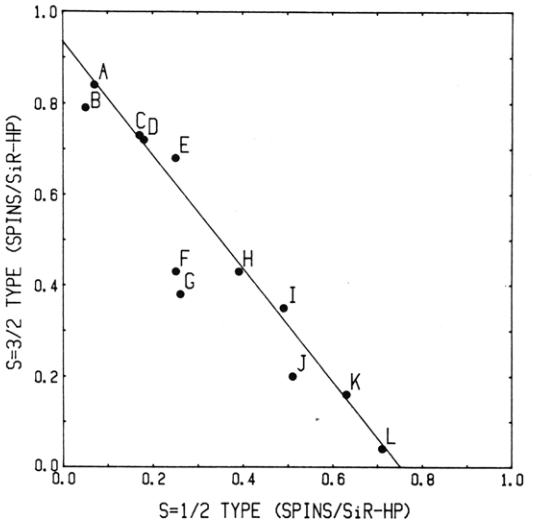

FIGURE 1: Inverse correlation between $S = {}^1/_2$ and $S = {}^3/_2$ type EPR signals in samples of photoreduced SiR-HP containing <0.1 spin/heme $g = 1.94$ type species. Anaerobic samples contained 72–100 μM SiR-HP in standard buffer, 10 mM EDTA, 25 μM Dfl, and the agents indicated. For details of the photoreduction process and conditions for measurement of each type of EPR species, see the text and Janick & Siegel (1982). The amounts (and g values) of the individual $S = {}^1/_2$ and $S = {}^3/_2$ type species found in each sample are given in Table I. In this figure all $S = {}^1/_2$ and $S = {}^3/_2$ species seen in a given sample have been summed to give a total spin per SiR-HP of each type of species. Additions to enzyme: A, 0.1 M $(Gdm)_2SO_4$; B, 0.1 M GdmCl; C, 5 mM GdmCl; D, 5 mM KCl; E, 0.5 mM KCl; F, 0.1 M sodium formate; G, 5 mM KBr; H, 0.1 mM KCl; I, 5 mM KF; J, 1 M urea; K, none; L, 40% (v/v) dimethyl sulfoxide.

amount of $S = {}^1/_2$ species present in our spin quantitation procedure.)

Strong Field Heme Ligands. (A) Cyanide. Siegel et al. (1982) have shown that while oxidized SiR-HP is relatively unreactive with added ligands, rates of ligand binding are markedly increased if the enzyme heme becomes reduced. Thus, CN^-, at 1 mM, binds to oxidized SiR-HP with a $t_{1/2}$ = 8.5 h, while binding to reduced SiR-HP occurs with $t_{1/2}$ = ca. 1 s. One mole of CN^- is bound per mol of heme, and the binding is essentially irreversible in standard buffer.

Photoreduction of SiR-HP with Dfl–EDTA in the presence of 1 mM KCN results in the production of a "fully reduced" enzyme–CN^- complex which is characterized optically by a split Soret band (401, 416 nm) and a broad band centered at 544 nm (Figure 2A). An EPR spectrum of the fully reduced SiR-HP–CN^- complex (Figure 3C) shows an intense $g = 1.94$ type signal quantitated at about 0.9 spin/heme. No EPR signals of the $S = {}^1/_2$ or $S = {}^3/_2$ types characteristic of uncomplexed fully reduced SiR-HP are detected in the spectrum of the fully reduced CN^- complex. The EPR spectrum of the fully reduced CN^- complex is shown in detail in Figure 4; its g values (determined by simulation of the spectrum) are given in Table I.

The fully reduced SiR-HP–CN^- complex can be incrementally oxidized by addition of small amounts of $Fe(CN)_6^{3-}$. The results of a titration of the fully reduced complex with $Fe(CN)_6^{3-}$ are shown in Figures 2 and 5. Addition of about 1 mol of $Fe(CN)_6^{3-}$/mol of heme (Figure 5) results in formation of an enzyme–CN^- complex with increased absorption in the Soret and a complex visible wavelength spectrum with a peak at 531 nm and a broad shoulder at 580 nm (Figure 2A). The transition between these two enzyme forms involves a set of isosbestic points at 364, 420, 536, and 578 nm. Figure 3B shows that the resulting "one-electron" SiR-HP–CN^- complex is EPR silent.

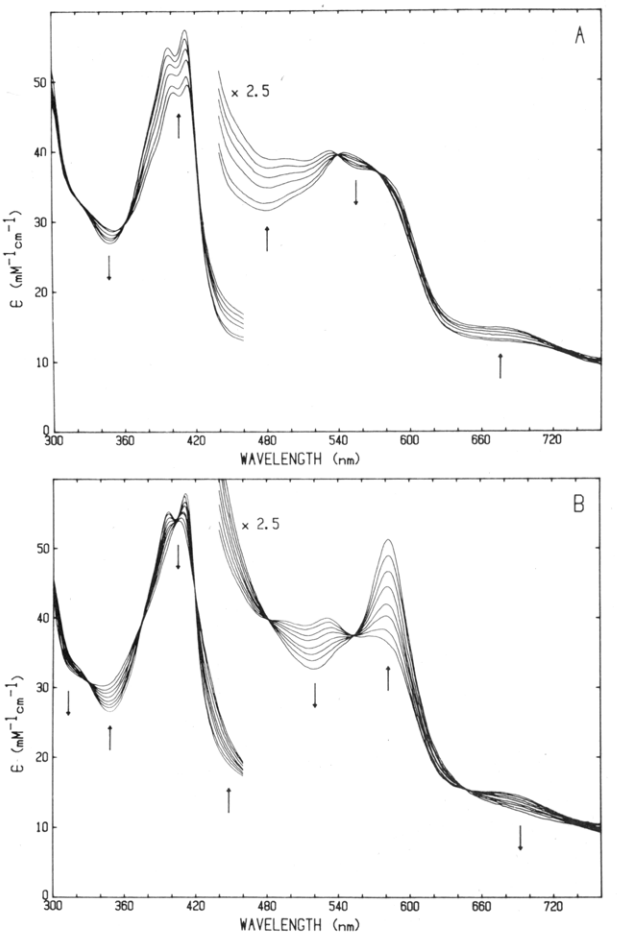

FIGURE 2: Optical spectra of fully reduced SiR-HP–CN^- complex upon reoxidation with ferricyanide. A solution containing 24 μM SiR-HP, 5 μM Dfl, 10 mM EDTA, 1 mM KCN, 15 ng/mL bovine liver catalase (Worthington), and 30 ng/mL bovine superoxide dismutase in standard buffer, total volume 0.5 mL, was made anaerobic in the double cuvette apparatus of Janick & Siegel (1982). A needle fitted to a gas-tight syringe containing 1.039 mM $K_3Fe(CN)_6$ in standard buffer was inserted into the apparatus, and the enzyme was then photoreduced until no further change in the optical spectrum occurred. Aliquots of the ferricyanide solution were added, and the optical spectrum was recorded after each addition. The direction of the spectral changes seen on oxidation at selected wavelengths is indicated by arrows. All spectra have been corrected for dilution. (A) Spectra obtained after addition of 0, 4, 8, 10, 12, and 14 μL, respectively, of ferricyanide solution. (B) Spectra obtained after addition of 16, 18, 20, 22, 24, 26, 28, and 32 μL, respectively, of ferricyanide solution. Further addition of ferricyanide did not alter the spectrum of the SiR-HP–CN^- complex.

Addition of a second mole of $Fe(CN)_6^{3-}$ per mole of heme[2] (Figure 5) results in production of a fully oxidized enzyme–CN^- complex. The optical spectrum of this oxidized complex

[2] Janick & Siegel (1982) have reported the presence of reducing equivalents of very negative potential in illuminated solutions of EDTA and Dfl in standard buffer. Such equivalents react more readily with $Fe(CN)_6^{3-}$ than do any of the reduced enzyme centers, and thus one observes a "lag" in the oxidation of reduced enzyme by added $Fe(CN)_6^{3-}$. Another set of reducing equivalents, of far more positive potential, is present in photoirradiated EDTA–Dfl solutions, this being the photoreduced forms of Dfl itself reported by Massey & Hemmerich (1978). The oxidation of the reduced Dfl can be followed spectrophotometrically in solutions from which enzyme has been omitted. The spectrophotometric changes observed on oxidation of the photoreduced Dfl are associated with the reduction of 1 mol of $Fe(CN)_6^{3-}$/mol of Dfl originally present. Given the relatively positive potential of the heme–CN^- complex in SiR-HP–CN^- (Siegel et al., 1982), it is likely that the extra 0.2 $Fe(CN)_6^{3-}$ utilized per SiR-HP–CN^- in the second phase of the titration of Figure 2 is due to oxidation of the 0.2 mol of reduced Dfl/mol of heme present in solution.

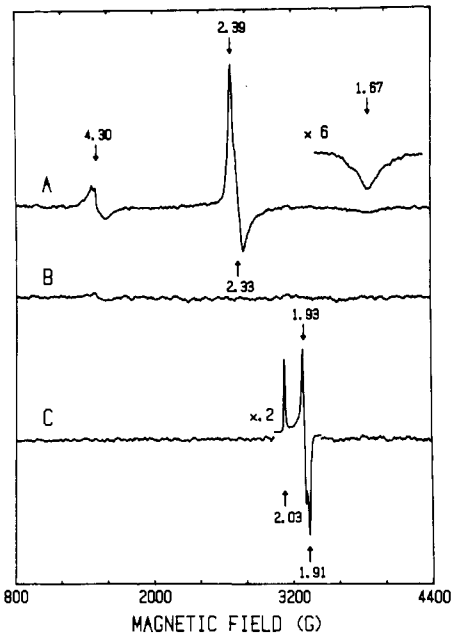

FIGURE 3: EPR spectra of the three oxidation states of SiR-HP–CN^- complex. The oxidized SiR-HP–CN^- complex (24 μM) was formed as described in Figure 2. Aliquots of the enzyme solution were then transferred to anaerobic EPR tubes, and these were then illuminated to rereduce the enzyme. The extent of reduction was monitored by measurement of the optical spectrum of each sample directly in the EPR tube by the procedure of Janick & Siegel (1982). After the desired optical spectrum was obtained, the sample was frozen in liquid N_2 for EPR analysis. EPR spectra were recorded at 9.12-GHz microwave frequency, 20 K temperature, and 10-mW microwave power. (A) Oxidized SiR-HP–CN^- (no illumination). (B) One-electron-reduced SiR-HP–CN^- (the enzyme was illuminated for 2 min to yield the optical spectrum corresponding to addition of 16 μL of $K_3Fe(CN)_6$ in Figure 2B). (C) Fully reduced SiR-HP–CN^- (the enzyme was illuminated for 5 min until no further optical change occurred; the optical spectrum was like that of the most reduced sample in Figure 1A).

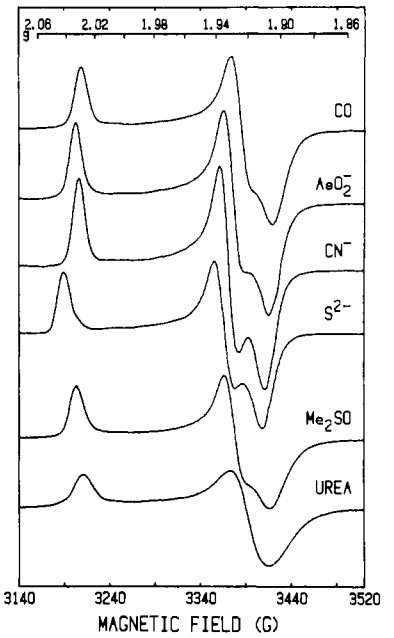

FIGURE 4: EPR spectra of SiR-HP photoreduced in the presence of 0.4 mM CO, 10 mM AsO_2^-, 1 mM CN^-, 5 mM S^{2-}, 60% (v/v) dimethyl sulfoxide, or 2 M urea. All samples contained 24 μM SiR-HP, 5 μM Dfl, 10 mM EDTA, and the indicated agent in standard buffer. Samples were illuminated under anaerobic conditions in EPR tubes until no further changes in optical spectra could be detected. EPR spectra were recorded at 9.12-GHz microwave frequency, 20 K temperature, 10-mW microwave power, and 10-G modulation amplitude. All EPR spectra have been normalized to constant double integral to facilitate comparison of spectra. For g values obtained by computer simulation of these spectra, see Table I.

exhibits a single Soret peak at 406 nm and an α-band maximum at 582 nm (Figure 2B). The EPR spectrum[3] of the oxidized SiR-HP–CN^- complex (Figure 3A) is characteristic of a low-spin ferriheme species with g = 2.39, 2.33, and 1.67. The spectrum shows no high-spin ferriheme. The transition between one electron and fully oxidized SiR-HP–CN^- complex exhibits a set of isosbestic points at 333, 372, 420, 477, 577, and 642 nm. The spectral changes seen in the course of the oxidative titration were completely reversed upon photoreduction of the oxidized SiR-HP–CN^- with Dfl–EDTA.

The combined EPR and optical data indicate that the first electron to be added to an oxidized SiR-HP–CN^- complex results in reduction of the CN^--ligated ferriheme, while the second electron results in reduction of the Fe_4S_4 center. The existence of an EPR-silent intermediate species, together with the presence of two nonoverlapping sets of isosbestic points in the optical spectra seen on oxidation of the fully reduced complex with $Fe(CN)_6^{3-}$, indicates that the reduction potentials for the heme and Fe_4S_4 centers in CN^--ligated SiR-HP are relatively far apart. Indeed, Siegel et al. (1982) have found potentials of −155 mV for the heme–CN^- Fe^{3+}/Fe^{2+} couple and −490 mV for the Fe_4S_4 (2+/1+) couple upon potentiometric titration of SiR-HP–CN^- complex in the presence of mediator dyes.

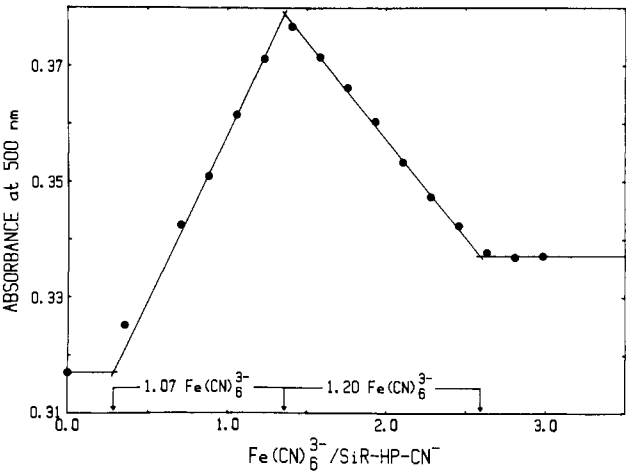

FIGURE 5: Titration of fully reduced SiR-HP–CN^- complex with ferricyanide. The absorbance at 500 nm (corrected for dilution) for each addition of $K_3Fe(CN)_6$ to fully reduced SiR-HP–CN^- complex in the experiment of Figure 2 is plotted as a function of the amount of ferricyanide added. The linear portions of the plot were fit by least-squares fits of the data in order to determine the beginning and end points of the two phases of the titration.

A difference optical spectrum between the fully reduced and one-electron-reduced SiR-HP–CN^- complexes is shown in Figure 6B. It can be seen that this optical transition, which involves reduction of an Fe_4S_4 center in enzyme in which the heme is already in the Fe^{2+} state, is considerably more complex than the generalized "bleaching" in the visible wavelength region normally seen on reduction of Fe_4S_4 center proteins. This result by itself indicates that there is electronic interaction

[3] Siegel et al. (1982) have reported slightly different values for the absorption maxima (404 and 581 nm) and g values (g = 2.39, 2.31, and 1.73) of the ferriheme–CN^- complex in SiR-HP–CN^-. Their spectra were obtained with enzyme reduced with MV^+ and reoxidized with either air or $Fe(CN)_6^{3-}$. The differences seen may reflect some interaction of methylviologen with the ligated enzyme.

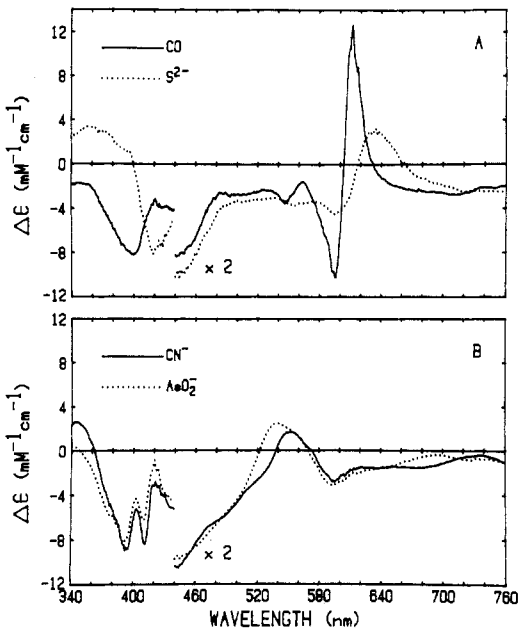

FIGURE 6: Optical difference spectra of reduced minus oxidized Fe_4S_4 centers in complexes of SiR-HP in which the heme is in the Fe^{2+} state. (A) CO (—) and S^{2-} (···) complexes. The difference spectrum for the CO complex was taken between the most reduced and oxidized species in the experiment of Figure 9. The difference spectrum for the S^{2-} complex was taken between that of the form 3 sample of Figure 7 (containing 0.5 spin/heme of $g = 1.94$ type EPR signal and no ferriheme after freezing) and that of an identical enzyme–S^{2-}–EDTA–Dfl solution illuminated for a shorter period of time (EPR spectrum contained 0.18 spin/heme of $g = 1.94$ type EPR signal and no ferriheme after freezing). The resulting difference spectrum of the S^{2-} complex has been multiplied by a factor of 3 to facilitate comparison with the other spectra. (B) CN^- (—) and AsO_2^- (···) complexes. The difference spectrum for the CN^- complex was between the fully reduced enzyme solution in the experiment of Figure 2 and that to which 16 μL of ferricyanide had been added (after correction of each spectrum for dilution). The difference spectrum for the AsO_2^- complex was between a solution containing 24 μM SiR-HP, 5 μM Dfl, 10 mM EDTA, and 10 mM $NaAsO_2$ in standard buffer which had been illuminated until no further change in optical spectrum could be detected, and the same solution to which 1.2 mol of $Fe(CN)_6^{3-}$/mol of SiR-HP had been added following the photoreduction. (The ferricyanide was added in small aliquots in a fashion like that in the experiment of Figure 2. Successive optical spectra obtained during the oxidation exhibited a common set of isosbestic points, indicating the presence of only two absorbing species. The most oxidized spectrum observed prior to loss of isosbesticity was used. Further addition of ferricyanide led to appearance of uncomplexed native oxidized SiR-HP.)

between the heme and the Fe_4S_4 centers in CN^--ligated SiR-HP.

(*B*) *Sulfide.* Like CN^-, sulfide binds much more rapidly to reduced than to oxidized enzyme. The affinity of SiR-HP for sulfide is much weaker than that for CN^-, although no precise measurements of dissociation constants have been made. Conversion of the majority of the enzyme to the sulfide complex required reduction of the enzyme in the presence of total sulfide concentrations of 1 mM or greater (at pH 7.7). The optical and EPR spectra of SiR-HP complexes with sulfide at different enzyme oxidation states are shown in Figures 7 and 8.

Prolonged photoreduction of enzyme in the presence of 5 mM sulfide resulted in production of "form 3" in those figures. This species exhibited optical maxima at 398 and 602 nm, and a $g = 1.94$ type of EPR signal shown in detail in Figure 4. No trace of the $S = {}^1/_2$ or $S = {}^3/_2$ type signals characteristic of free fully reduced SiR-HP was detected. Although integration of this signal yielded only 0.5 spin/SiR-HP, it is quite possible

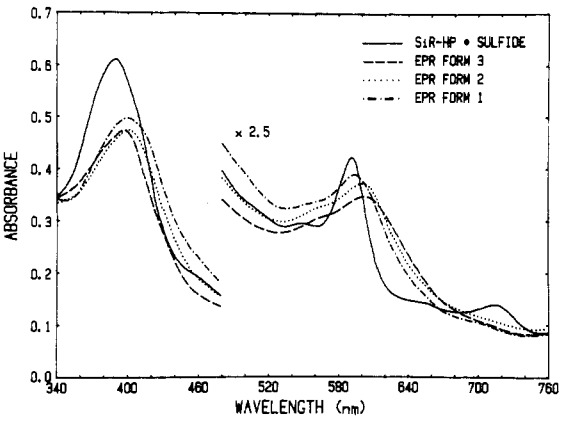

FIGURE 7: Optical spectra of three oxidation states of SiR-HP–S^{2-} complex. Anaerobic solutions containing 30 μM SiR-HP, 6 μM Dfl, 10 mM EDTA, and 5 mM Na_2S in standard buffer were illuminated in EPR tubes and the optical spectrum of each solution recorded. (—) Enzyme to which S^{2-} had been added, no illumination (no complex formation detected). (---) Form 3 enzyme–S^{2-} complex: The solution was photoreduced until no further optical change was detected (this required 10 min of illumination). (···) Form 2 enzyme–S^{2-} complex: The solution was photoreduced for 4 min. (-·-) Form 1 enzyme–S^{2-} complex: O_2-saturated standard buffer was added to an EPR tube containing form 3 complex until no further change in optical spectrum could be detected. The spectrum is corrected for dilution.

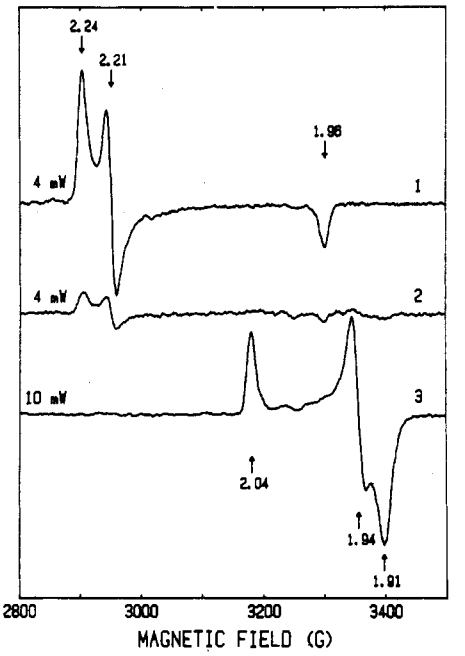

FIGURE 8: EPR spectra of three oxidation states of SiR-HP–S^{2-} complex. The enzyme samples of Figure 7 were frozen in liquid N_2, their EPR spectra were recorded at 9.12-GHz microwave frequency, 20 K temperature, 10-G modulation amplitude, and the microwave powers indicated.

that reduction of the S^{2-}-ligated enzyme was incomplete. As seen in Figure 4 and Table I, the line shape and g values of the reduced Fe_4S_4 center EPR spectrum are different when SiR-HP is complexed with sulfide as opposed to CN^-.

When O_2-saturated buffer was added to form 3 enzyme until no further optical change occurred, an oxidized enzyme–sulfide complex was produced ("form 1"), with optical absorption maxima at 400 and 594 nm and an EPR spectrum characteristic of low-spin ferriheme with $g = 2.24$, 2.21, and 1.96. Integration of this signal yielded 0.7 spin/SiR-HP. As with the oxidized SiR-HP–CN^- complex, there was no trace of any high-spin ferriheme in the EPR spectrum of oxidized enzyme–sulfide complex, a result which indicates that complex

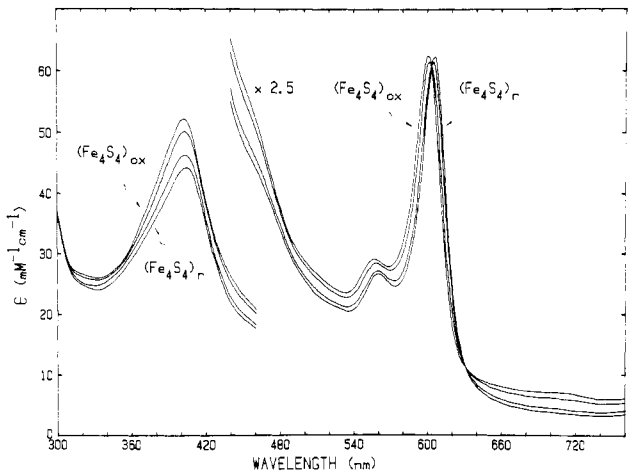

FIGURE 9: Optical spectra of SiR-HP–CO complex in which the Fe_4S_4 center is oxidized or reduced. An anaerobic solution containing 24 μM SiR-HP, 5 μM Dfl, 10 mM EDTA, and 0.4 mM CO in standard buffer was illuminated for short periods of time followed by 10-min incubations in the dark (to allow CO complexation with the ferroheme to occur) until the 715-nm charge transfer band characteristic of the native enzyme ferriheme was absent. (This process required 3-min total illumination and 50-min total incubation.) The resulting optical spectrum is labeled "$(Fe_4S_4)_{ox}$" in the figure. The solution was then illuminated for 60-s periods and optical spectra recorded after each illumination. The spectrum observed after 3 min of total illumination of the $(Fe_4S_4)_{ox}$ SiR-HP–CO complex did not change on continued illumination and thus represents that of the fully reduced complex. It is labeled "$(Fe_4S_4)_r$" in the figure. Addition of air to the fully reduced SiR-HP–CO complex yielded a species with an optical spectrum identical with that of $(Fe_4S_4)_{ox}$ in the figure.

formation was complete in these experiments.

A partially reduced enzyme–sulfide complex yielded optical and EPR spectra labeled "form 2" in Figures 7 and 8. In the sample obtained, there were 0.11 spin/heme of form 1 and 0.04 spin/heme of form 3. The great majority of the enzyme in the intermediate reduction state is diamagnetic, like the enzyme–CN^- complex reduced by one electron. It would appear that this enzyme oxidation state contains ferroheme and oxidized Fe_4S_4 in the sulfide complex as well.[4]

Figure 6A shows that the optical difference spectrum between fully reduced and one-electron-reduced SiR-HP–sulfide complexes, like that of the CN^- complex, is strongly suggestive of electronic interaction between heme and Fe_4S_4 center in the sulfide-ligated enzyme.

(*C*) *Carbon Monoxide.* As shown by Murphy et al. (1974), CO binds tightly to either free or enzyme-bound Fe^{2+} (but not Fe^{3+}) siroheme. The rate of CO dissociation from its complex with SiR-HP is slow ($t_{1/2}$ = 2 h).

Figure 4 shows an EPR spectrum of the reduced Fe_4S_4 center in the photoreduced enzyme–CO complex (1.0 spin/heme) together with spectra of reduced enzyme complexed to several other ligands. All of these are of the g = 1.94 type. From the g values (Table I) and line shape of the CO complex spectrum as compared to the other spectra shown, it is clear that the EPR spectrum of the Fe_4S_4 center is sensitive to the nature of the ligand present.

Optical spectra of SiR-HP–CO complexes in which the Fe_4S_4 center is either reduced, partially oxidized, or fully oxidized (as determined by EPR spectra of parallel samples with the same optical spectra) while the ferroheme remains reduced (the only oxidation state which will bind CO) are shown in Figure 9. These "hemochromogen"-type spectra are characteristic of ferrous siroheme–CO complexes whether free or enzyme bound (Siegel et al., 1978; Stolzenberg et al., 1981). Thus we may conclude that there is a ferroheme–CO complex in each of these spectra. While the fully reduced enzyme–CO complex yields the g = 1.94 signal characteristic of reduced Fe_4S_4 centers, the oxidized complex is EPR silent. Figure 9 shows that there is a distinct bathochromic shift of 4 nm in the α-band maximum, as well as a decline in absorbance in the Soret region, when the Fe_4S_4 center of SiR-HP containing ferroheme–CO is reduced. This result is particularly evident in the difference spectrum shown for the SiR-HP–CO complex in Figure 6A. The change in the spectrum of ferriheme–CO on reduction of the Fe_4S_4 center is apparent, providing strong evidence for the presence of electronic interaction between the SiR-HP heme and Fe_4S_4 prosthetic groups in CO-complexed enzyme.

(*D*) *Arsenite.* Arsenite is a nonreducible competitive inhibitor of sulfite reduction by SiR (Siegel et al., 1974). AsO_2^- appears to have little or no affinity for oxidized enzyme.

Photoreduction of SiR-HP with 10 mM AsO_2^- leads to the sequential formation of two optical species which closely resemble the optical spectra of the one- and two-electron-reduced SiR-HP–cyanide complexes. The more oxidized of these enzyme–AsO_2^- species is EPR silent, indicating the presence of ferroheme and oxidized Fe_4S_4, while the fully reduced enzyme–AsO_2^- complex exhibits a g = 1.94 type of EPR signal (shown in Figure 4; g values in Table I), indicating reduction of the Fe_4S_4 center. Quantitation of this signal yielded 0.85 spin/SiR-HP. Figure 6 shows that the optical difference spectrum between the fully reduced and EPR silent enzyme–AsO_2^- complexes is similar to that for the transition between the two- and one-electron-reduced SiR-HP–CN^- complexes. Thus, we may infer that the ferroheme in the SiR-HP–AsO_2^- complexes is low spin. The EPR and optical difference spectra of the enzyme–AsO_2^- complex shown in Figures 4 and 6 strongly indicate the presence of heme–Fe_4S_4 interaction in the presence of this ligand.

Potential Weak Field Heme Ligands. (*A*) *Chloride.* Ferriheme chloride complexes ($S = {}^5/_2$) have been characterized for iron isobacteriochlorins as well as for other hemes (Stolzenberg et al., 1981). Figure 10 shows EPR spectra of SiR-HP fully photoreduced in the presence of KCl.[5] It can

[4] If form 2 enzyme is in thermodynamic equilibrium with forms 1 and 3, and as in unligated enzyme, the heme and Fe_4S_4 centers are reduced independently, one can calculate a potential difference of 135 mV between the two centers in the enzyme–S^{2-} complex, assuming that 1.0 spin per heme represents the maximal spin concentration of each center. (If the maximal spin concentration is assumed not to exceed the integrations of form 1 or 3, the centers would be 104 mV apart.) Siegel et al. (1982) have reported that the centers are 335 mV apart in CN^--ligated SiR-HP, and Janick & Siegel (1982) have shown that the centers differ by 65 mV in unligated SiR-HP. Thus, the relative ease of reduction of the heme and Fe_4S_4 centers in SiR-HP is strongly dependent on the type of ligand bound to the enzyme.

[5] Although the optical and EPR spectra of the chloride complex of oxidized free siroheme are significantly different from the spectra of oxidized native SiR-HP (Siegel et al., 1973), we have found no alteration in the EPR spectrum of oxidized enzyme or in the optical spectrum of any oxidation state of SiR-HP upon photoreduction in the presence of KCl concentrations ranging from 0.1 mM to 1 M (Figure 11A). The spectra of enzyme subjected to reduction in the presence of such KCl concentrations and subsequently reoxidized with O_2 or $Fe(CN)_6^{3-}$ were identical with those of enzyme treated with the same procedures in the absence of KCl (essentially identical with untreated native SiR-HP). Although high concentrations of KCl were found to inhibit the MV^+–SO_3^{2-} reductase activity of SiR-HP, 50% inhibition being observed at 0.4 M KCl, this effect is probably due to a generalized anion effect on the enzyme similar to that reported by Siegel et al. (1974) for the MV^+–SO_3^{2-} reductase activity of NADPH–SiR hemoflavoprotein complex. There was no effect at all of 5 mM KCl on SiR-HP catalytic activity (as measured in the standard assay containing 1 mM SO_3^{2-}).

Table I: EPR Spectra Observed in Fully Reduced SiR-HP in the Presence of Added Agents[a]

addition to standard buffer	type of EPR signal observed [spins per SiR/HP (g values)]			total spins per SiR-HP
	$g = 1.94$	$S = 1/2$	$S = 3/2$	
none	0.03 (2.036, 1.931, 1.911)	0.63 (2.53, 2.29, 2.07)	0.09 (4.82, 3.39, ca. 2.0) 0.07 (5.23, 2.80, ca. 2.0)	0.82
0.1 mM KCl	0.03 (2.036, 1.931, 1.911)	0.33 (2.53, 2.29, 2.07) 0.06 (2.71, 2.30, ca. 2.03)	0.43[b] (5.08, 2.55, ca. 2.0) (5.30, 2.71, ca. 2.0) 0.03 (4.82, 3.39, ca. 2.0)	0.88
5 mM KCl	0.07[b] (2.049, 1.934, 1.913) (2.036, 1.931, 1.911)	0.18 (2.71, 2.30, ca. 2.03)	0.40 (5.08, 2.55, ca. 2.0) 0.32 (5.30, 2.71, ca. 2.0)	0.95
1.0 M KCl	0.09[b] (2.049, 1.934, 1.913) (2.036, 1.931, 1.911)	0.15 (2.71, 2.30, ca. 2.03)	0.38 (5.08, 2.55, ca. 2.0) 0.26 (5.30, 2.71, ca. 2.0)	0.90
5 mM KF	0.06[b] (2.043, 1.934, 1.911) (2.036, 1.931, 1.911)	0.49 (2.58, 2.27, 2.08)	0.35 (5.23, 2.80, ca. 2.0)	0.90
5 mM KBr	0.07[b] (2.047, 1.935, 1.914) (2.036, 1.931, 1.911)	0.26 (2.53, 2.29, 2.07)	0.19 (4.82, 3.39, ca. 2.0) 0.19 (5.23, 2.80, ca. 2.0)	0.71
0.1 M sodium formate	0.07[b] (2.046, 1.938, 1.915) (2.036, 1.931, 1.911)	0.25 (2.77, 2.33, ca. 2.04)	0.37 (4.72, 3.04, 2.17) 0.06 (5.23, 2.86, ca. 2.0)	0.76
5 mM GdmCl	0.03 (2.036, 1.931, 1.911)	0.17[b] (2.71, 2.30, ca. 2.03) (2.53, 2.29, 2.07)	0.33 (4.88, 3.31, 2.08) 0.40[b] (5.08, 2.55, ca. 2.0) (5.30, 2.71, ca. 2.0)	0.93
0.1 M GdmCl	0.05[b] (2.049, 1.934, 1.913) (2.036, 1.931, 1.911)	0.05 (2.71, 2.30, ca. 2.03)	0.42 (5.30, 2.71, ca. 2.0) 0.37 (5.08, 2.55, ca. 2.0)	0.89
0.1 M $(Gdm)_2SO_4$	0.05 (2.036, 1.931, 1.911)	0.07 (2.53, 2.29, 2.07)	0.84 (4.88, 3.31, 2.08)	0.96
1.0 M urea	0.11[b] (2.028, 1.919, 1.908) (2.036, 1.931, 1.911)	0.50 (2.53, 2.29, 2.07)	0.18 (4.90, 3.09, ca. 2.0)	0.79
2.0 M urea	0.50 (2.028, 1.919, 1.908)[c]	0.09 (2.53, 2.29, 2.07)	0.05 (4.90, 3.09, ca. 2.0)	0.64
3.3 M urea	0.34 (2.028, 1.919, 1.908)[c]	0	0	0.34
40% (v/v) Me_2SO	0.10 (2.035, 1.930, 1.908)	0.71 (2.65, 2.26, ca. 2.04)	0.04 (4.72, 3.48, ca. 2.0)	0.85
60% (v/v) Me_2SO	0.80 (2.033, 1.928, 1.905)[c]	0	0	0.80
1 mM KCN	0.82 (2.031, 1.933, 1.907)[c]	0	0	0.82
5 mM Na_2S	0.50 (2.042, 1.934, 1.910)[c]	0	0	0.50
0.4 mM CO	1.02 (2.029, 1.925, 1.904)[c]	0	0	1.02
10 mM $NaAsO_2$	0.85 (2.034, 1.929, 1.907)[c]	0	0	0.85

[a] For details of procedures used for generating photoreduced samples and for recording EPR spectra of individual signal types, see the text and the legends to relevant figures. [b] The figure given represents the sum of the two species whose g values are given on this and the next line. It was not possible to accurately quantitate the individual species. [c] g values obtained by simulation of the EPR spectrum using the program of Lowe (1978).

be seen that the complex set of resonances seen in the native enzyme is replaced by a new set of resonances, predominately of the $S = 3/2$ type, in 5 mM KCl. The changes can be summarized as follows (see Table I): (1) The majority $S = 1/2$ type species (0.63 spin/heme) present in native reduced enzyme is markedly reduced in intensity in 5 mM Cl^- (to 0.06 spin/heme), and its g values are shifted. (2) The two relatively minor $S = 3/2$ type species present in native enzyme (total 0.16 spin/heme) are replaced by new $S = 3/2$ type signals[6] (a relatively sharp signal with $g = 5.30$, 2.71, and ca. 2.0, and a broad signal with $g = 5.08$, 2.55, and ca. 2.0) of similar intensity to one another, which taken together now comprise the majority type of signal (0.7 spin/heme) in the reduced enzyme. There is only a small increase in the amount of the $g = 1.94$ type EPR species over that found in the reduced native enzyme (from 0.03 to 0.07 spin/heme) in 5 mM KCl.[7]

When one examines the points for various concentrations of KCl in Figure 1 (compare points K, H, E, and D), it can be seen that the effect of KCl in causing a shift of the $S = 1/2$ to the $S = 3/2$ type of EPR signals was approximately 50% complete with 0.1 mM KCl and was over 90% completed with 0.5 mM KCl. Since the SiR-HP concentration in these experiments was 72 μM, 1.3 Cl^- per heme were sufficient to convert about half of the enzyme to the "chloride"-type reduced form. This indicates that a highly specific and high-affinity binding of 1 or 2 mol of Cl^-/mol of enzyme is responsible for the changes.

(B) Other Halides and Formate.[8,9] The effects of these

[6] Deconvolution of the EPR spectrum of SiR-HP reduced in the presence of 50 mM KCl was achieved by taking advantage of the fact that the various component species displayed different temperature and relaxation behavior. The $S = 1/2$ type signals were markedly saturated at 6 K, 150 mW, unlike the $S = 3/2$ type signals [see Janick & Siegel (1982)]. Subtraction of an EPR spectrum taken at 6 K, 150 mW, from a spectrum recorded at 6 K, 10 mW (with correction for the dependence of the $S = 3/2$ type signal intensity on the square root of the power), afforded a spectrum of the $S = 1/2$ type species at 6 K. The resonance features in the $g = 5.0$–5.4 region in spectra taken at 6 K clearly involve at least two species: a sharp feature at $g = 5.3$ and a broad underlying feature at a slightly smaller g value. At 13 K, the sharp portion of the resonance at $g = 5.3$ was found to be markedly diminished as compared to the 6 K spectrum, leaving a much broader resonance centered about $g = 5.1$. Subtraction of the 13 K spectrum from the 6 K spectrum (with elimination of the features due to the previously obtained $S = 1/2$ type species) produced the spectrum shown in Figure 10 of the sharp $S = 3/2$ component, with $g = 5.30$, 2.71, and ca. 2.0. The high field portion of this spectrum was not well resolved. Subtraction of the resulting spectrum from the 6 K, 150 mW spectrum (again with elimination of the features due to $S = 1/2$ type species) produced the broad spectrum shown in Figure 10 with resonances at $g = 5.08$, 2.55, and ca. 2.0.

[7] When the KCl concentration was raised from 5 mM to 1 M, there was a slight decrease in the intensity of both the $S = 3/2$ and $S = 1/2$ type EPR signals in reduced SiR-HP, and an increased amount of a "$g = 19.4$" type signal with g values slightly different from those found in native enzyme (Table I). Even at 1 M KCl, however, the $g = 1.94$ type signal was a small minority species, representing only 0.1 spin per heme.

[8] Reduction of SiR-HP in the presence of 5 mM KF, 5 mM KBr, or 100 mM sodium formate (which forms a high-spin ferriheme complex with the cytochrome a_3 moiety of cytochrome oxidase; Babcock et al., 1976) had no effect on the optical spectra of any of the enzyme oxidation states or on the EPR spectrum of oxidized enzyme (even after a cycle of reduction and air reoxidation in the presence of the anion). All of the effects detected with these agents were fully reversed following removal of the anion by gel filtration.

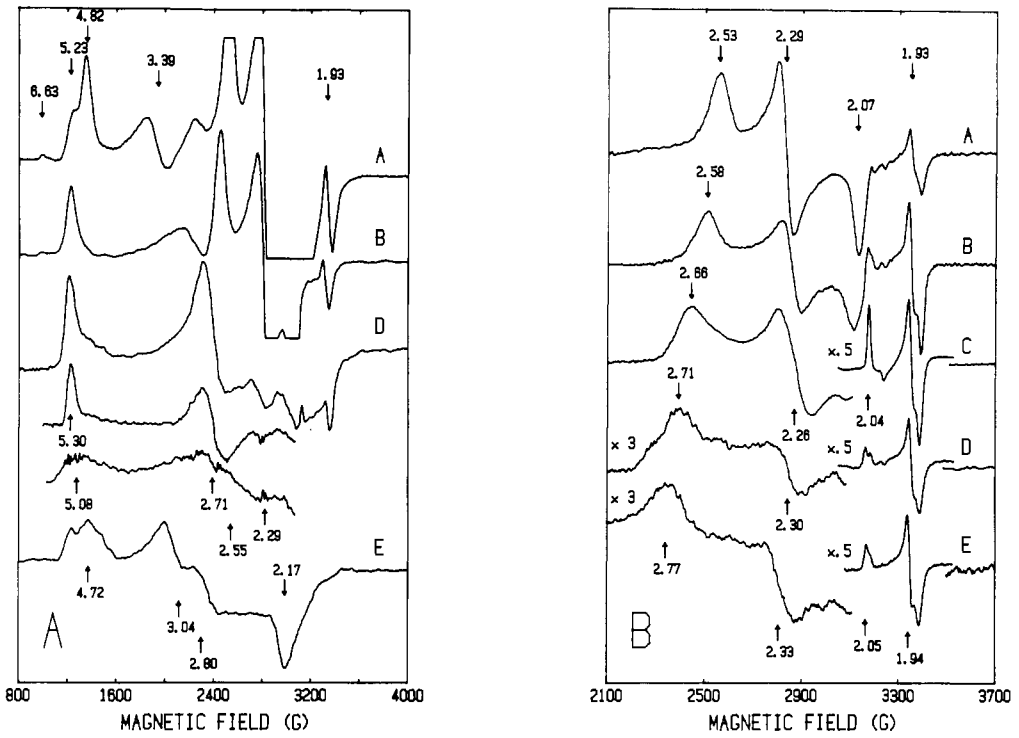

FIGURE 10: EPR spectra of SiR-HP fully reduced in the presence of potential weak field heme ligands. Anaerobic solutions in standard buffer containing 10 mM EDTA and either 210 μM SiR-HP and 40 μM Dfl (spectrum A), 72 μM SiR-HP, 25 μM Dfl, and 5 mM KF (spectrum B), 72 μM SiR-HP, 25 μM Dfl, and 40% (v/v) dimethyl sulfoxide (spectrum C), 72 μM SiR-HP, 25 μM Dfl, and 5 mM KCl (spectrum D), or 100 μM SiR-HP, 25 μM Dfl, and 100 mM sodium formate (spectrum E) were prepared in EPR tubes, and the solutions were photoreduced until no further changes in optical spectrum could be detected. The solutions were frozen in liquid N_2 and EPR spectra recorded at 9.12-GHz microwave frequency. (Panel A) $S = {}^3/_2$ type species: Spectra A, B, and D were recorded at 8 K temperature and 100-mW microwave power, conditions at which the $S = {}^1/_2$ and $g = 1.94$ type species are highly saturated, whereas the $S = {}^3/_2$ type species are not saturated. The features of the $S = {}^1/_2$ type species were permitted to go off scale. The two spectra shown immediately below spectrum D are those derived for the $g = 5.30$ (upper) and $g = 5.08$ (lower) species by the procedure indicated in footnote 6 of the text. Spectrum E was recorded at 5 K and 50-mW microwave power in order to better show the position of the high-field features of the $S = {}^3/_2$ type spectrum (by more fully saturating the overlapping $S = {}^1/_2$ type species). (Panel B) $S = {}^1/_2$ and $g = 1.94$ type species: Spectra were recorded at 20 K temperature and 50-mW microwave power. Under these conditions the spectra of the $S = {}^3/_2$ species are not observed. The spectra have been normalized to constant enzyme concentration in panel B but not in panel A.

agents on the EPR spectra of fully reduced SiR-HP were similar to those of 5 mM KCl in that there was an increased amount of $S = {}^3/_2$ type species and a decreased amount of $S = {}^1/_2$ type species as compared to native enzyme (Table I).[10] The g values observed for these species were, however, different from those seen with KCl. Figure 10 shows EPR spectra of reduced SiR-HP in the presence of F^- and formate. The spectra observed with the latter ion were particularly shifted with respect to those seen in the native enzyme, the g_1 feature of the $S = {}^1/_2$ signal being shifted to 2.77 in the presence of formate (the largest g value yet seen for this type of signal in SiR-HP) and the g_3 feature of the major $S = {}^3/_2$ species exhibiting the unusually high value of 2.17 in formate-containing solutions.[11]

Potential Chaotropic Agents. (A) Dimethyl Sulfoxide. Although Me_2SO is commonly used as an agent which can perturb protein structure [see Cammack (1975)], Mashiko et al. (1978) and Zorbrist & LaMar (1978) have shown that Me_2SO can also act as a weak field heme ligand, the resulting ferriheme reportedly being in the $S = {}^5/_2$ state in model compounds. In keeping with the possibility that Me_2SO could exert distinct types of effects on a heme-containing protein, we have found that Me_2SO, depending on its concentration, produces two different effects on the EPR spectrum of fully reduced SiR-HP (Table I): One, seen when the proportion of Me_2SO added to standard buffer is 40% (v/v) or less, results in conversion of nearly all of the $S = {}^3/_2$ signals seen in the native reduced enzyme species to a single species of the $S = {}^1/_2$ type. The g values of the latter species (as well as the residual $S = {}^3/_2$ species of Me_2SO-containing enzyme solutions) are shifted from those seen in the native reduced enzyme

[9] Photoreduction of SiR-HP in the presence of 100 mM KBr occurred without alteration of the optical spectrum of the reduced enzyme (recorded at 23 °C) from that seen with native reduced SiR-HP. However, after the sample was frozen in liquid N_2 for EPR analysis, the emerald green color characteristic of reduced native SiR-HP, which normally remains on freezing, changed to a red color in the 100 mM KBr-treated enzyme. EPR spectra of the latter enzyme sample showed the presence only of a $g = 1.94$ type signal (0.8 spin per heme), with g values like those of the species with the greatest g_1 seen in the presence of 5 mM KBr. A similar freezing-dependent alteration in the color of the enzyme, accompanied by the presence only of $g = 1.94$ type signal in the EPR spectrum, was found with SiR-HP photoreduced in the presence of 100 mM SCN^- or OCN^-. We have not yet determined whether or not these effects are reversible. Interestingly, the potent lyotropic anion ClO_4^-, at 100 mM, had no effect at all on the EPR spectrum of photoreduced SiR-HP.

[10] It should be noted (see Table I) that photoreduction of SiR-HP in the presence of 5 mM KBr or 100 mM sodium formate appeared to be incomplete even when irradiation periods of 2 h were used.

[11] Although only small amounts of $g = 1.94$ type EPR signals were detected in SiR-HP reduced in the presence of 5 mM KCl, 5 mM KF, 5 mM KBr, or 100 mM sodium formate, the increased amount of this signal in each of these cases was associated with the appearance of a new set of signals of the $g = 1.94$ type. In all cases, the new signal exhibited its g_1 feature at a value significantly greater than that seen for this type of signal with native SiR-HP or any of its complexes with well-defined low-spin heme ligands. The interpretation of this result is not clear at present.

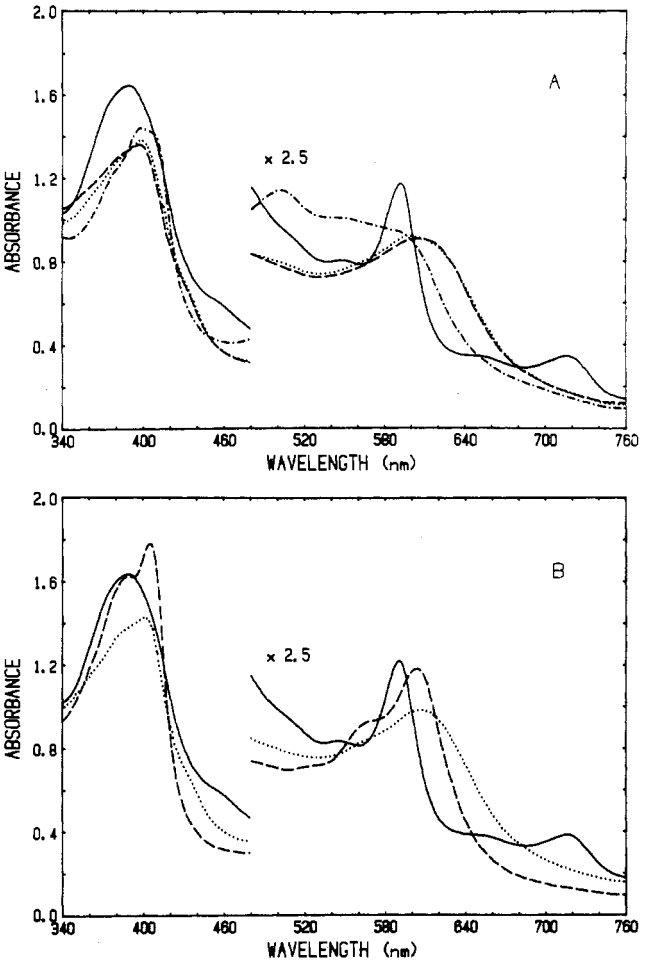

FIGURE 11: Optical spectra of SiR-HP photoreduced in the presence of various agents. Anaerobic solutions containing 72 μM SiR-HP, 25 μM Dfl, 10 mM EDTA, and the indicated agents were photoreduced in EPR tubes until no further changes in optical spectra could be detected. (A) (—) Oxidized enzyme in 60% (v/v) Me_2SO prior to illumination [spectra obtained for oxidized enzyme prior to illumination in the presence of either no added agents, 50 mM KCl, 1 M KCl, 5 mM KF, 100 mM sodium formate, 1 M or 2 M urea, or 20 or 40% (v/v) Me_2SO were identical with this spectrum]. (---) Enzyme photoreduced in the presence of 50 mM KCl [spectra obtained for enzyme fully reduced in the presence of no additions, 1 M KCl, 5 mM KF, 100 mM sodium formate, or 20% (v/v) Me_2SO were identical with this spectrum]. (···) Enzyme photoreduced in the presence of 40% Me_2SO. (-·-) Enzyme photoreduced in the presence of 60% (v/v) Me_2SO. (B) (—) Oxidized enzyme in 2 M urea prior to illumination. (···) Enzyme photoreduced in the presence of 1 M urea. (---) Enzyme photoreduced in the presence of 2 M urea.

(Figure 10). The effect could be detected at Me_2SO concentrations as low as 1% and was nearly complete in 20% Me_2SO. (It should be noted that Me_2SO is the only agent found to date by us which promotes conversion of $S = {}^3/_2$ to $S = {}^1/_2$ type species in SiR-HP.) The second effect, seen when the proportion of Me_2SO is increased to 60% (v/v) and greater, involves replacement of these signals by a single species of the $g = 1.94$ type. This signal, amounting to 0.8 spin/heme, has g values which are different than those observed with the previously described low-spin heme ligands (Figure 4). As seen in Table I, the two effects of Me_2SO are partially overlapping, since the amount of $g = 1.94$ type signal is increased from 0.03 spin/heme in native enzyme to 0.10 spin/heme in 40% Me_2SO.

The effects of Me_2SO on the optical spectra of fully reduced SiR-HP are shown in Figure 11A. The optical spectrum in 20% Me_2SO is like that seen in native reduced SiR-HP. The spectrum in 60% Me_2SO is markedly different, showing a series of broad overlapping visible wavelength bands (with a maximum at 490 nm) and a complex Soret region (with a shoulder at 408 nm). The latter spectrum resembles that of low-spin ferroheme complexes, and Mössbauer spectroscopy[12] has indicated that the ferroheme in SiR-HP fully reduced in 60% Me_2SO is indeed $S = 0$. (The optical spectrum of reduced enzyme in 40% Me_2SO is a mixture of a small amount of the 60% Me_2SO spectrum with a 20% Me_2SO spectrum, in agreement with the presence of increased $g = 1.94$ signal in the 40% Me_2SO enzyme.)

Oxidized SiR-HP (or enzyme reduced and then reoxidized) in 40% or 60% Me_2SO exhibits the same optical spectrum as native enzyme, although the ferriheme g values are shifted slightly (to $g = 6.54$, 5.35, and 1.99). Thus, the effects of 60% Me_2SO (like those of AsO_2^-) in altering the spin state of ferroheme are reversed when the heme is in the ferric state. If the 40% or 60% Me_2SO is removed from SiR-HP (reduced and then reoxidized) by gel filtration, the EPR and optical spectra of the resulting enzyme are substantially like those of native enzyme in all oxidation states. Although the MV^+–SO_3^{2-} reductase activity of SiR-HP is totally inhibited if 40% or 60% Me_2SO is included in the assay itself, enzyme which has been reduced in the presence of either concentration of Me_2SO is fully active if diluted into assay mixtures which do not contain Me_2SO.

We may conclude that Me_2SO at low concentrations perturbs the EPR spectrum of fully reduced SiR-HP without changing the paramagnetic nature ($S = 1$ or 2) of the ferroheme. In contrast, Me_2SO at higher concentrations causes a change in the ferroheme spin state to $S = 0$, with a concomitant appearance of the $g = 1.94$ type signal characteristic of this heme spin state in the reduced enzyme. It is by no means necessary to presume that the ligand responsible for the spin-state change in the presence of such a highly structure perturbing solvent as Me_2SO is Me_2SO itself. It could easily be an endogenous heme ligand which gains access to the heme as a result of a conformational change induced in the enzyme at high Me_2SO concentrations.

(*B*) *Guanidinium Salts and Urea.* Guanidinium salts, at low concentration (5–200 mM in guanidinium ion), cause shifts in the EPR spectra of fully reduced SiR-HP which are similar to those observed in the presence of halides and formate, i.e., an increased amount of $S = {}^3/_2$ type species, a decrease in the amount of $S = {}^1/_2$ type species, and shifts in the g values of one or more of these species (Table I). Under conditions in which these effects are observed, there are no changes in the optical spectra of SiR-HP in any oxidation state, the enzyme remains fully active, and the EPR signals of native enzyme are regained if the guanidinium salts are removed by gel filtration and the enzyme rereduced.

As reported by Janick & Siegel (1982), the EPR spectrum of SiR-HP fully reduced in the presence of 100 mM $(Gdm)_2SO_4$ exhibits a sharp, highly rhombic $S = {}^3/_2$ type signal with $g = 4.88$, 3.31, and 2.08, which integrates to 0.84 spin/heme (Table I), and only small amounts of signals of the $S = {}^1/_2$ (0.07 spin/heme) and $g = 1.94$ (0.05 spin/heme) types. A control solution of enzyme reduced in the presence of 100 mM K_2SO_4 showed little change in the overall proportion of the various types of signal observed in the native enzyme spectrum. Thus the effect observed in 100 mM $(Gdm)_2SO_4$ is primarily a guanidinium and not a sulfate effect.

GdmCl, at 5 mM, also leads to production of the "$g = 4.88$" species seen in $(Gdm)_2SO_4$ and loss of the $S = {}^1/_2$ type species

[12] J. A. Christner, E. Munck, P. A. Janick, and L. M. Siegel, unpublished results.

(Table I). However, additional $S = {}^3/_2$ type resonances in the $g = 5.1$–5.3 region, of the type seen with KCl, are also observed in 5 mM GdmCl. Raising the GdmCl concentration to 100 mM results in elimination of the $g = 4.88$ guanidinium-induced signal from the reduced SiR-HP EPR spectrum and its replacement by a spectrum similar to that seen in 5 mM KCl.

Urea, at 1 M, has relatively minor effects on the optical (Figure 11B) or EPR (Table I) spectra of SiR-HP in any oxidation state. The observed $S = {}^3/_2$ type species in the fully reduced enzyme remains at about 0.2 spin/heme, but the g values are those of a single species. There is also a significantly increased amount of $g = 1.94$ type signal (to 0.11 spin/heme), with g values like those seen in 2 M urea, and a concomitant decrease in the $S = {}^1/_2$ type signal. These changes (except for the increased $g = 1.94$ signal) are largely reversed on removal of the urea.

At 2 M, urea drastically alters the EPR (Table I) and optical (Figure 11B) spectra of fully reduced SiR-HP. Only small amounts of the $S = {}^3/_2$ and $S = {}^1/_2$ type EPR signals remain, and a new majority species of the $g = 1.94$ type (0.52 spin/heme) is observed. This signal (Figure 4) is considerably broader than those seen in other enzyme forms with the $g = 1.94$ type EPR signal. The optical bands in fully reduced enzyme in 2 M urea are seen at 404 and 602 nm. Although the optical and EPR spectra of oxidized SiR-HP in 2 M urea are like those of native enzyme (except for a shift in ferriheme g values to 6.57, 5.39, and 1.99) and such enzyme is fully active if the urea is removed, the spectra of SiR-HP which had been reduced in the presence of 2 M urea and then reoxidized are substantially altered (there being little high-spin ferriheme present, a small low-spin ferriheme signal at $g = 2.38$ and 2.30, g_3 not determined, and an intense signal at $g = 4.3$), and enzyme so treated is inactive if the urea is removed. The "2 M urea" optical spectra and EPR signals persist if the enzyme is reduced in the absence of urea, indicating that reduction in the presence of 2 M urea causes an irreversible change in the structure of the enzyme active center. Similar results to those obtained with 2 M urea were obtained if the concentrations of guanidinium salts were raised to 0.5 M. At higher urea or guanidinium concentrations, photoreduction of SiR-HP led to progressive loss of all EPR signals from the enzyme as it apparently underwent further denaturation.

Discussion

Christner et al. (1981) have shown by Mössbauer spectroscopy that the siroheme and Fe_4S_4 centers are exchange coupled in oxidized SiR-HP, a result which implies the existence of a chemical link between the Fe atom of the heme and an Fe atom of the Fe_4S_4 cluster. Janick & Siegel (1982) showed that reduction of SiR-HP results in loss of the high-spin ferriheme EPR signal characteristic of the oxidized enzyme and the appearance of novel sets of EPR signals termed $S = {}^1/_2$ type (0.63 spin/heme) and $S = {}^3/_2$ type (0.16 spin/heme) in the fully reduced enzyme. Only a small amount of the classical $g = 1.94$ signal expected for isolated reduced Fe_4S_4 centers was observed (0.03 spin/heme). The novel signals, which were present in constant ratio in several different enzyme preparations, were interpreted as arising from an exchange interaction between the reduced Fe_4S_4 ($S = {}^1/_2$) and an $S = 1$ or 2 ferroheme in the chemically linked centers of the reduced enzyme. Mössbauer spectroscopic data on fully reduced SiR-HP was consistent with this interpretation.

EPR signals of the $S = {}^1/_2$ type, but with somewhat different g values, have been seen in the ferredoxin–sulfite (Krueger & Siegel, 1982b) and ferredoxin–nitrite[13] reductases of spinach, both of which contain one siroheme and one Fe_4S_4 center at the active site of catalysis (Krueger & Siegel, 1982a; Lancaster et al., 1979). The reduced nitrite reductase also exhibits EPR signals of the $S = {}^3/_2$ type.[13] Preliminary Mössbauer spectra of oxidized spinach nitrite reductase, cited by Christner et al. (1981), showed that the heme and Fe_4S_4 centers are exchange coupled in that enzyme as well as in *E. coli* SiR-HP.

Siegel et al. (1982) and Lancaster et al. (1979), in their attempts to demonstrate the presence of a reducible Fe_4S_4 center in *E. coli* SiR-HP and spinach nitrite reductase, respectively, found that an EPR signal of the classical $g = 1.94$ type could be produced quantitatively upon reduction of the enzymes (with reduced methylviologen) in the presence of CO or CN^-. Krueger & Siegel (1982b) have reported similar results with spinach sulfite reductase. The apparent midpoint potentials for the Fe_4S_4 center of SiR-HP differed by 70 mV depending on whether CO or CN^- was present.

These findings have been considerably amplified by the results of the present work. Thus, we have found that addition to reduced SiR-HP of any agent which causes the ferroheme to become low spin such as the strong field ligands CO, cyanide, sulfide, or arsenite or perturbants such as 60% Me_2SO (which presumably allows the reversible binding of an endogenous strong field ligand to the ferroheme) results in the concomitant appearance of the $g = 1.94$ EPR signal in the fully reduced enzyme. That the ferroheme is indeed low spin in the presence of these agents is suggested by the optical spectra observed, the presence of $S = {}^1/_2$ ferriheme EPR signals in the CN^- and sulfide complexes on reoxidation, and the fact that the Mössbauer parameters of the CO-, CN^--, or S^{2-}-complexed ferroheme in reduced SiR-HP and of the ferroheme in enzyme reduced in 60% Me_2SO are consistent with an $S = 0$ spin state.[12] (Mössbauer spectroscopy has not been performed on AsO_2^--complexed SiR-HP.)

Since the perturbation induced in the $S = {}^1/_2$ reduced Fe_4S_4 center EPR spectrum by the presence of a magnetic $S = 1$ or 2 ferroheme, such as that present in native reduced SiR-HP, would be removed if the ferroheme became $S = 0$, it is not surprising that low-spin heme ligands result in the observation of only $g = 1.94$ type EPR signals in reduced SiR-HP. This result, however, in no way implies that the chemical bond required to mediate magnetic exchange interaction between the heme and Fe_4S_4 centers in native enzyme has been broken when the heme has become low spin. In fact, the ligand-dependent differences observed in the g values and exact line shapes of the $g = 1.94$ type EPR signals seen in reduced SiR-HP in the presence of low-spin ferroheme ligands, as well as the variation of the observed Fe_4S_4 center midpoint potential of ligated SiR-HP on the nature of the low-spin ferroheme ligand present, strongly suggest that there is interaction between the Fe_4S_4 center and the ligated heme in such enzyme complexes. Additional evidence for such interaction in ligated SiR-HP derives from the optical difference spectra corresponding to reduction of the Fe_4S_4 center in enzyme where the heme is maintained in the low-spin ferrous state. In such spectra, the changes observed are markedly ligand dependent and clearly involve perturbations in the electronic properties of the heme. A similar influence of Fe_4S_4 center oxidation state on the siroheme optical spectrum was observed in the "unligated" native SiR-HP by Janick & Siegel (1982).

Preliminary data on Mössbauer spectroscopy[12] of oxidized SiR-HP complexes with CN^- and sulfide show that there

[13] J. O. Wilkerson, P. A. Janick, and L. M. Siegel, unpublished results.

appears to be a significant broadening of the oxidized Fe_4S_4 spectrum induced by the $S = {}^1/_2$ ferroheme, a result which is consistent with the presence of exchange coupling between the two prosthetic groups in these ligated forms of SiR-HP. As argued for native oxidized SiR-HP by Christner et al. (1981), exchange coupling requires a chemical link between the two enzyme prosthetic groups.

It seems reasonable to suppose, as a working hypothesis to explain the observed magnetic and optical interactions, that the siroheme Fe is linked to a corner of the Fe_4S_4 cluster by a bridging ligand. Although the sulfur atom of one of the cysteine ligands of the Fe_4S_4 cluster represents an obvious candidate for such a bridging ligand in the native enzyme, it must be emphasized that we have no data on the chemical nature of the endogenous ligand at present.

Since the siroheme molecule is essentially flat with axial binding sites on either side of the Fe atom, and since there is good evidence that binding of ligands such as CO, CN^-, S^{2-}, and SO_3^{2-} involves the heme Fe (Siegel et al., 1982; this work), there are obviously two possibilites for the stereochemistry of binding of such exogenous ligands with respect to the Fe_4S_4 cluster: The added ligand could bind to the heme Fe trans to the Fe_4S_4 cluster, with the observed coupling between the two prosthetic groups maintained by the same endogenous ligand present in the native enzyme. Alternatively, the added ligand could displace the endogenous bridging group and itself serve to bridge the two enzyme prosthetic groups. Such cis addition of the added ligand is essentially a ligand substitution reaction on one of the Fe atoms of the Fe_4S_4 cluster. Such substitution reactions have been shown to occur readily in $Fe_4S_4(SR)_4$ model compounds, with replacement of RS^- by halides, CN^- (Job & Bruice, 1975), $RCOO^-$ (Johnson & Holm, 1978), and NO (Gall et al., 1974), as well as $R'S^-$ (Que et al., 1974). Transition metal complexes involving bridging ligands such as CN^- (Shriver, 1966), CO (Alich et al., 1972), halides (Mulay & Boudreaux, 1976), S^{2-} (Holm & Ibers, 1976), or NO (Connelly, 1972) are well-known in the literature.

Cytochrome oxidase possesses an antiferromagnetically coupled heme a_3 and copper. On the basis of EXAFS analyses, Powers et al. (1981) have proposed that these two centers are bridged by a cysteine sulfur atom. Tweedle et al. (1978) have shown that the exchange interaction seen in the oxidized enzyme is maintained on binding of CN^- to the heme Fe. As with SiR-HP, it remains an open question as to whether CN^- (and substrate) binds trans to the coupled centers or between them in cytochrome oxidase. It is of interest of course that both cytochrome oxidase and the siroheme–Fe_4S_4 enzymes (sulfite and nitrite reductases) are catalysts of reactions in which several electrons (four to six) must be rapidly transferred to bound substrate without release of potentially very reactive compounds of intermediate oxidation state from the enzyme. It seems probable that the presence of coupled electron transferring centers in these enzymes can be useful in either facilitating the rate of reduction of bound substrate or holding onto the substrate and intermediates in a particularly tight fashion during the reduction process, or both.

The EPR spectra observed with reduced SiR-HP in the presence of potential weak field heme ligands such as halides, formate, and Me_2SO and with low concentrations of certain perturbants such as guanidinium salts and Me_2SO show that there is a facile interconversion between the two types of EPR spectrum ($S = {}^1/_2$ and $S = {}^3/_2$ type) characteristic of the native fully reduced enzyme. With each type of spectral species, a variety of g values can be obtained depending on the nature of the added agent. In all cases, the enzyme remains active (either in the presence of the agent or upon its removal), and the observed EPR effects are fully reversed upon removal of the agent.

Although, in the case of Cl^-, the observed effects on the EPR spectrum may be correlated with a rather high-affinity binding of halide to SiR-HP, there is no compelling evidence that such binding must occur at the heme. However, if Cl^- and similar ligands did indeed bind to the ferroheme of reduced SiR-HP, they could potentially induce a change in spin state of the native enzyme ferroheme (e.g., from $S = 1$ to $S = 2$), thus accounting for the altered EPR spectrum observed. However, the fact that the optical spectra of fully reduced SiR-HP are substantially the same in enzyme yielding either the $S = {}^1/_2$ or $S = {}^3/_2$ type of EPR spectrum suggests that the observed effects may not in fact be due to alterations in heme spin state. An alternate possibility that the changes in EPR spectrum reflect rather minor alterations in the strength of the coupling interaction between the prosthetic groups in SiR-HP cannot be excluded at present. Hatfield (1976) had discussed the effects of small changes in bound angles and distances on the strength and even the sign of exchange interactions in bridged metal complexes.

Registry No. SiR, 9029-35-0; CN^-, 57-12-5; S^{2-}, 18496-25-8; CO, 630-08-0; AsO_2^-, 17306-35-3; dimethyl sulfoxide, 67-68-5; Cl^-, 16887-00-6; F^-, 16984-48-8; Br^-, 24959-67-9; $(Gdm)_2SO_4$, 594-14-9; GdmCl, 50-01-1; urea, 57-13-6; sodium formate, 141-53-7; siroheme, 52553-42-1.

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Effect of Solubilization on Adenosine 5′-Triphosphate Induced Calcium Release from Purified Sarcoplasmic Reticulum Calcium Adenosinetriphosphatase†

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ABSTRACT: ATP-induced Ca^{2+} release from the purified sarcoplasmic reticulum Ca^{2+}-ATPase has been monitored in several different ATPase environments. Arsenazo III was used as a Ca^{2+} indicator in stopped-flow experiments and was shown to detect the early burst in Ca^{2+} transport, slower steady-state transport, and release of Ca^{2+} from fragmented sarcoplasmic reticulum. ATP-induced rapid release of Ca^{2+} followed by a slower rebinding step could be demonstrated for purified Ca^{2+}-ATPase in leaky vesicles if the reaction was slowed by lowering the pH to 6.1 and by including dimethyl sulfoxide in the reaction medium. At a dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$) to protein weight ratio of 0.2, a detergent concentration too low for solubilization to occur, ATP-induced Ca^{2+} release occurred more rapidly than for native leaky membranes, whereas the rebinding step was slower. In contrast, no Ca^{2+} release was observed for any soluble preparation. The kinetics of Ca^{2+} release was studied under conditions where the ATPase was monomeric or aggregated, and also in the presence of added phospholipid. The ATPase was shown to be monomeric by sedimentation equilibrium measurements in the presence of Ca^{2+}, ADP, and β,γ-methylene-ATP at a $C_{12}E_8$ to protein weight ratio of 2.0. It is concluded that solubilization of the Ca^{2+}-ATPase may result in uncoupling of ATP hydrolysis from ATP-induced Ca^{2+} release.

The Ca^{2+}-ATPase[1] from sarcoplasmic reticulum has recently been shown to exhibit cooperative binding of two calcium ions per phosphorylation site (Inesi et al., 1980; Verjovski-Almeida & Silva, 1981) although earlier studies did not reveal cooperativity (Meissner, 1973; Ikemoto, 1975). Since this Ca^{2+} binding level represents only one Ca^{2+} per ATPase polypeptide in native membranes, the observed pumping stoichiometry of two Ca^{2+} per ATP hydrolyzed suggests that only half of the total ATPase molecules transport Ca^{2+} at any given time and that the functional unit of the ATPase is at least a dimer (Inesi et al., 1980; Verjovski-Almeida & Silva, 1981; Ikemoto et al., 1981; Froehlich & Taylor, 1976). This conclusion thus supports the proposal that the Ca^{2+}-ATPase is aggregated in its native membrane (Ikemoto et al., 1981). In contrast, there are data showing that monomeric ATPase in detergent solution retains many properties of the native enzyme (Dean & Tanford, 1978; Jorgensen et al., 1978; Dean & Gray, 1980). Furthermore, structural studies indicate that a single Ca^{2+}-ATPase polypeptide possesses all of the necessary structures for Ca^{2+} transport (MacLennan et al., 1980; Green et al., 1980). However, the solubilized, monomeric enzyme lacks negative cooperativity of ATP hydrolysis (Inesi et al., 1980; Dean & Tanford, 1978), cooperativity of Ca^{2+} binding (Verjovski-Almeida & Silva, 1981), and the ability to form a covalent phosphoenzyme intermediate from inorganic phosphate (Nestruck-Goyke & Hasselbach, 1981).

Solubilization of the Ca^{2+}-ATPase results in several experimental advantages, although it eliminates the possibility of assaying Ca^{2+} transport since there is no aqueous compartment in detergent solution for Ca^{2+} accumulation. However, a closely related activity which should be measurable in detergent solution is ATP-induced Ca^{2+} release from the ATPase. Ikemoto (1976) used the Ca^{2+}-sensitive dye arsenazo III to observe rapid, ATP-induced release of Ca^{2+} from purified ATPase in leaky vesicles. More recently Watanabe et

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; $C_{12}E_8$, dodecyl octaoxyethylene glycol monoether; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; ATPase, adenosinetriphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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