Proton NMR of *Escherichia coli* Sulfite Reductase: Studies of the Heme Protein Subunit with Added Ligands†

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ABSTRACT: The heme protein subunit of sulfite reductase (SiR-HP; M_r 64 000) from *Escherichia coli* as isolated contains the isobacteriochlorin siroheme exchange-coupled to a [4Fe-4S] cluster in the 2+ oxidation state. SiR-HP in the presence of a suitable electron donor can catalyze the six-electron reductions of sulfite to sulfide and nitrite to ammonia. Paramagnetic ^{1}H NMR was used to study the low-spin complexes of SiR-HP formed by binding the exogenous inhibitor cyanide or the substrates sulfite and nitrite. As a model, the cyanide complex of purified siroheme was also prepared. The NMR spectrum of isolated ferric low-spin siroheme–CN is consistent with spin density being transferred into the a_{2u} molecular orbital, an interaction which is symmetry-forbidden in porphyrins. The pattern of proton NMR shifts observed for isolated ferric low-spin siroheme–CN is very similar to those obtained for the protein–cyanide complex. NMR spectra of the cyanide complex of SiR-HP were obtained in all three accessible redox states. The pattern of hyperfine shifts observed for the one-electron and two-electron reduced cyanide complexes is typical of those seen for [4Fe-4S] clusters in the 2+ and 1+ oxidation states, respectively. Resonances arising from the β-CH_2 protons of cluster cysteines have been assigned for all complexes studied utilizing deuterium substitution. The cyanide-, sulfite-, and nitrite-ligated states possessed an almost identically shifted upfield cluster cysteine resonance whose presence indicates that covalent coupling exists between siroheme and cluster in solution. Data are also presented for the existence of a secondary anion binding site, the occupancy of which perturbs the oxidized SiR-HP NMR spectrum, where binding occurs at a rate much faster than that of ligand binding to heme.

The active site of *Escherichia coli* sulfite reductase heme protein subunit (SiR-HP)[1] consists of a [4Fe-4S] cluster exchange-coupled to a siroheme prosthetic group (Christner et al., 1981; Janick & Siegel, 1982). A model of the active site has been proposed (Janick & Siegel, 1982) in which one cluster Fe is covalently bridged to the siroheme Fe by the sulfur of a cysteinal cluster ligand. X-ray crystallographic data (McRee et al., 1986) are consistent with this model, although it lacks sufficient resolution to identify the bridging ligand. Recently, we have examined the proton NMR of unligated SiR-HP and have presented data consistent with cysteine acting as the bridging ligand (Kaufman et al., 1993b).

On the distal side of the siroheme, opposite the [4Fe-4S] cluster, the X-ray crystal structure of oxidized SiR-HP does not show significant electron density (McRee et al., 1986). This may therefore be the site of binding for a number of exogenous ligands, including the substrates sulfite and nitrite and inhibitory agents such as cyanide and CO. In all complexes studied to date, ligand binding appears to involve the heme prosthetic group (Siegel et al., 1982). A role for siroheme in the binding of substrate at the SiR-HP active site was originally inferred from the observation that typical heme ligands such as CN and CO elicited optical and EPR changes characteristic of heme axial ligation (Siegel et al., 1973). The substrates sulfite and nitrite also elicit changes in the optical spectrum of SiR-HP consistent with axial binding to the heme, although the majority of species generated in these reactions are EPR silent (Janick et al., 1983). Magnetic susceptibility studies on the sulfite complex of oxidized SiR-HP have identified it as being $S = {}^1/_2$ in the frozen state (Day et al., 1988). The binding of CN and sulfite was shown to be mutually exclusive (Siegel et al., 1982). There are two potential sites for the binding of exogenous ligands to the heme: either at the distal side of the heme or at the proximal side by replacing the endogenous bridging ligand. In the latter case, the exogenous ligand may act as the bridge between cluster and heme.

Here we use paramagnetic proton NMR spectroscopy to characterize the low-spin complexes of SiR-HP in solution and compare the results with those derived using the model low-spin isobacteriochlorin complex, siroheme–CN. This is the first reported NMR spectrum of an isolated ferric low-spin isobacteriochlorin.[2] The cyanide complex of the heme protein was studied in all three redox states. The pattern of hyperfine shifts observed for the one-electron and two-electron reduced cyanide complexes is typical of those seen for [4Fe-4S] clusters in the 2+ and 1+ oxidation states. We can readily demonstrate that the sulfite, nitrite, and oxidized cyanide complexes of SiR-HP are all $S = {}^1/_2$ in solution, as evidenced

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[1] Abbreviations: SiR, sulfite reductase; SiR-HP, oxidized sulfite reductase heme protein subunit; SiR-HP^{1-}, half-reduced SiR-HP; SiR-HP^{2-}, fully reduced SiR-HP; SiR-HP–CN, oxidized sulfite reductase heme protein subunit bound to cyanide; SiR-HP–NO_2, oxidized sulfite reductase heme protein subunit bound to nitrite; SiR-HP–SO_3, oxidized sulfite reductase heme protein subunit bound to sulfite; SB, standard buffer 100 mM KP_i (pH 7.7); SBE, standard buffer with 100 μM EDTA; OEiBC, octaethylisobacteriochlorin; [4Fe-4S]$^{2+/1+}$, oxidized, reduced four iron–four sulfur cluster; HiPiP, high-potential iron–sulfur protein; RR, resonance Raman; OEP, octaethylporphyrin; δ_{con}, contact shift; KP_i, potassium phosphate; Dlfl, deazalumiflavin.

[2] Isobacteriochlorins differ from porphyrins in that they possess two adjacent partially saturated pyrroline rings. Siroheme, an iron isobacteriochlorin, is found at the active site of all sulfite reductases studied so far.

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by their narrowed line widths and longer relaxation times compared to those in the high-spin NMR spectrum. Cluster cysteine resonances were identified both by the presence of anti-Curie temperature dependence and by use of SiR-HP into which β-CD_2-cystine has been incorporated. All ferric $S = {}^1/_2$ siroheme enzyme complexes examined possessed a similarly shifted upfield cluster resonance, a result which is interpreted as demonstrating that covalent coupling is maintained between heme and cluster in SiR-HP in solution in the presence of exogenous ligands. Interaction between the two prosthetic groups has been proposed to be of functional significance in facilitating the rapid transfer of electrons to the enzyme-bound substrate (Siegel & Wilkerson, 1989). The maintenance of the chemical linkage between cluster and heme while exogenous ligands are bound is therefore crucial to this model.

MATERIALS AND METHODS

E. coli NADPH-sulfite reductase was purified from cell pastes according to the published procedures (Siegel et al., 1973; Siegel & Davis, 1974; Kaufman et al., 1993b). SiR-HP was obtained from plasmid overproducers of either the holoenzyme or the heme protein subunit (Wu et al., 1991b). Following isolation and dialysis vs SBE, the protein was concentrated by Amicon filtration to 110 μM and stored in liquid nitrogen until used. Concentrations were determined spectrophotometrically using an $\epsilon_{591} = 18.1\ mM^{-1}\ cm^{-1}$ (Siegel et al., 1982).

EPR spectra were taken with a Bruker ER200D spectrometer at a nominal temperature of 15–20 K, a modulation amplitude of 10 G, an operating frequency of 9.47–9.48 GHz, and a modulation frequency of 100 kHz. Spin concentrations were determined from spectra recorded under nonsaturating conditions either by double integration and comparison with a Cu(II)-EDTA standard or by integration of an absorption-type peak and comparison with a standard of known concentration. A Hewlett-Packard 9825A computer equipped with a 9874A digitizer and a 7225A graphics plotter was used for integration and plotting of spectra.

NMR spectra were run in double-precision mode over 16K data points on either a General Electric GN 300-MHz spectrometer with a 12-bit ADC, a General Electric GN 500-MHz spectrometer with a 16-bit ADC, or a Varian 600-MHz spectrometer using a 16-bit ADC in the Duke Magnetic Resonance Spectroscopy Center. Samples were loaded into 5-mm NMR tubes with typical volumes of 400–600 μL. The relaxation delay time consisted only of the water presaturation time unless otherwise noted. The 90° pulse time was 12.5 μs on the GE 300 and 8–9 μs on the GE 500 or Varian 600 spectrometer. Pulse times less than 90° were used in most experiments to obtain uniform excitation over the large sweep widths used (Kaufman et al., 1993a). All chemical shifts are given in parts per million from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) referenced against the residual solvent signal. A line broadening of 15 Hz was applied to all low-spin samples to minimize noise. Protein samples were exchanged into D_2O by being repeatedly (four or five times) concentrated to about 1 mL on an Amicon filtration device equipped with a PM30 membrane and then diluted to 10 mL with 99.9% D_2O (pD 7.7) SB.

All solid and liquid chemicals were of reagent or analytical grade and, unless noted otherwise, were not purified further. The DL-(3,3,3′,3′-d_4)-cystine and the EDTA-d_{12} were both obtained from Cambridge Isotope Laboratories.

The protocol used to prepare anaerobic SiR-HP was as outlined previously (Kaufman et al., 1993b), with reduction being achieved photochemically using 10 mM EDTA-d_{12} plus either deazaflavin or deazalumiflavin at one-fifth the protein concentration (Massey & Hemmerich, 1978; Janick & Siegel, 1982). After being rendered anaerobic, the protein samples were transferred utilizing a gastight syringe to an NMR tube which had been previously flushed with argon and was fitted with a rubber septum.

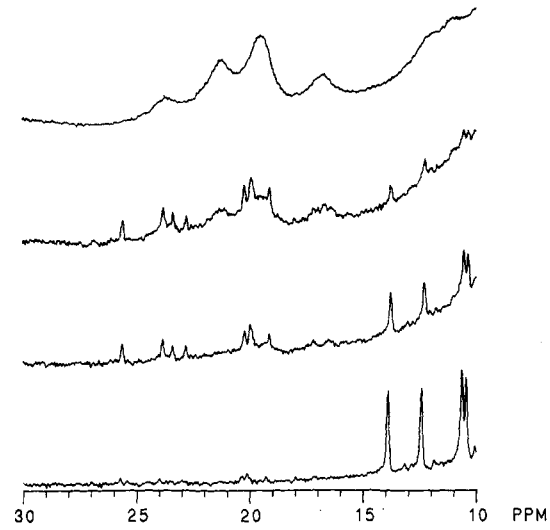

FIGURE 1: 300-MHz NMR spectra in D_2O at 15 °C of the downfield region during a cyanide titration of siroheme at 15 °C. Spectra from top to bottom were obtained immediately after 0, 5.05, 10.7, or 38.9 mM potassium cyanide was added at pD 6.0.

RESULTS

Low-Spin Siroheme–Cyanide Complex. The low-spin ferric cyanide complex of siroheme ($S = {}^1/_2$) was formed by reacting KCN with the free heme at pH 6.0. The EPR (g values of 2.37 and 1.78; 0.82 spin per heme) and optical (Soret 384, alpha 578 nm, ratio 5.1) spectra are consistent with previously published results (Kang et al., 1987). Varying the pH between 6.0 and 7.7 had little effect on the optical spectrum of the cyanide complex. Estimates of the K_d for cyanide binding at pH 6.0 range from 1.1 to 1.4 mM with slow cyanide binding ($t_{1/2}$ = hours). The optical spectra generated upon the addition of KCN failed to show a clean isosbestic point. The NMR spectra show the presence of an intermediate low-spin species at low cyanide concentrations (Figure 1); this intermediate may be the monocyanide complex (with or without bound water or hydroxide at the sixth position). Alternatively, this intermediate spectral form could represent a species in a state of aggregation different from that of the final product. The final NMR spectrum illustrated in Figure 2 gives 21 resonances with a total intensity of 29 protons as shown in Table I, where the labeling scheme used for siroheme peaks is the same as previously reported (Kaufman et al., 1993b). Assignments can be made to classes of protons based on T_1 measurements and coupling observed in P-COSY experiments (Figure 3). Peaks L and O can be assigned to the pyrroline methyl protons based on intensity, small isotropic shift, and high T_1 values. Peaks R–U have significantly shorter T_1s than any other protons present, and on the expectation that the meso positions are closest to the iron, they can be attributed to meso protons. On the basis of symmetry, the meso protons may be further split into two classes (Table I). Peaks A and B show coupling to each other and to peak K, as do peaks C and D to peak J. Peaks A–D are tentatively assigned to propionate groups bound to the pyrrole rings. The four doublets seen between 6.95 and 5.23 ppm are assigned to acetate groups.

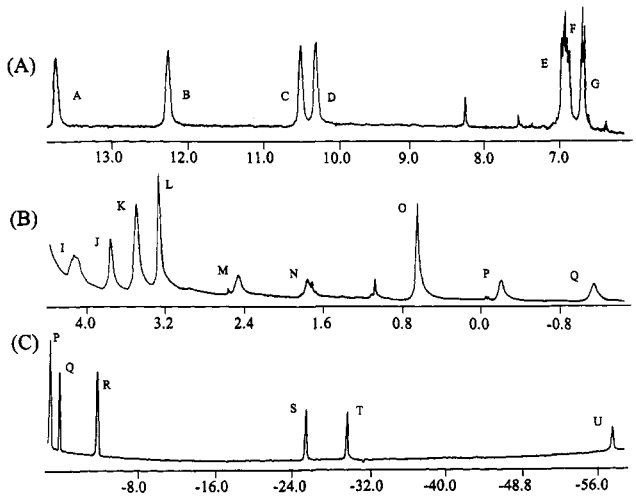

FIGURE 2: ^{1}H NMR (15 °C) spectrum of 1.1 mM siroheme–cyanide (pD 6.0) in 99.9% D_2O. Sections A, 13.8 to 6.1 ppm, B, 4.3 to −1.6 ppm, and C 0 to −60 ppm were each scaled individually to the largest peak present. The spectrum was obtained on a General Electric 500-MHz FT-NMR spectrometer using an 8-μs pulse over a spectral width of 37 000 Hz collected with 16K data points. A total of 1024 scans were obtained in 1 h. The residual water signal was suppressed by a presaturation pulse of 600 ms. Line broadening of 2 Hz was applied to minimize noise.

Table I: ^{1}H NMR Data for $S = {}^1/_2$ Fe(III) (pH 6.0) Siroheme–CN Complex

label[a]	ppm[b]	int (str)[c]	T_1 (ms)	cross peaks	assignment[d]
A	13.67	1 (S)	57	B, K	17a or 13a
B	12.17	1 (S)	74	A, K	17a or 13a
C	10.47	1 (S)	62	D, J	17a or 13a
D	10.27	1 (S)	66	C, J	17a or 13a
E	6.95	1 (D)	73	G	18a or 12a
F	6.88	1 (D)	68	H	18a or 12a
G	6.72	1 (D)	45	E	18a or 12a
H	5.23	1 (D)		F	18a or 12a
I	4.12	1 (S)	61		
J	3.75	2 (S)	97	C, D	17b or 13b
K	3.49	4 (S)[e]		A, B	17b or 13b
L	3.21	3 (S)	69		2 or 7 methyl
M	2.44	1 (S)		N, Q	
N	1.74	1 (S)	63	M, P	
O	0.61	3 (S)	55		2 or 7 methyl
P	−0.26	1 (S)	51	M, Q	
Q	−1.21	1 (S)	45	N, P	
R	−5.07	1 (S)	20.1		5 or 20 meso
S	−26.27	1 (S)	14.2		10 or 15 meso
T	−30.40	1 (S)	13.9		10 or 15 meso
U	−58.1	1 (S)	10.7		5 or 20 meso

[a] All labels are for the peaks given in Figure 2. [b] Peak position for the sample at 15 °C. Sample referenced against DSS. [c] Intensity (structure). [d] Labeling scheme used for siroheme peaks can be found in Kaufman et al. (1993b). [e] Resolves into a peak intensity two and two singlets at higher temperature.

The temperature dependence of the siroheme–cyanide complex was unusual in that four resonances showed anti-Curie temperature dependence and the intercepts of many peaks at $1/T = 0$ deviated significantly from the diamagnetic region (Table I; Figure 4). The presence of anti-Curie temperature dependence suggests that aggregation is present despite the absence of any concentration effect over the concentration range (750 μm–1.8 mM) studied. However, the marked similarity of spectral form in free siroheme–CN and SiR-HP–CN suggests that whether or not aggregation is present in solution, the siroheme–CN spectrum remains a useful model. Other possible causes of anti-Curie temperature dependence will be discussed below.

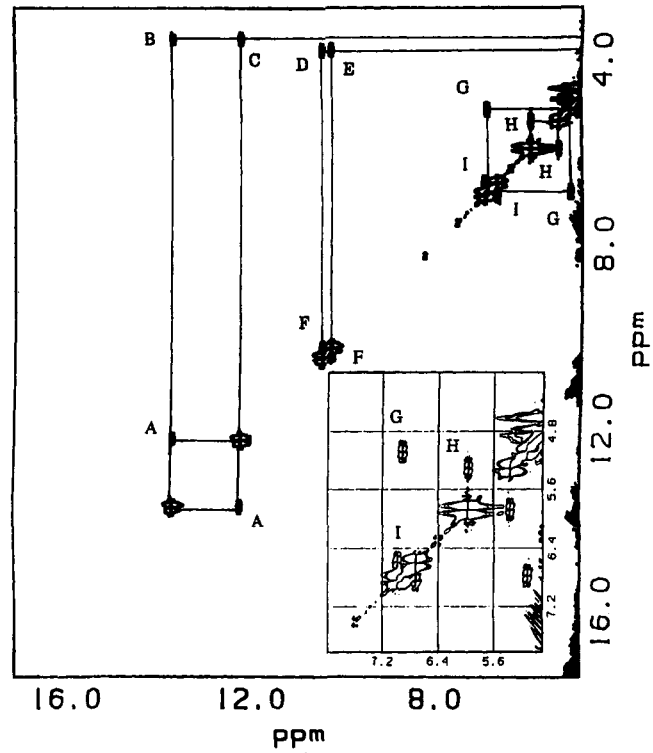

FIGURE 3: 500-MHz ^{1}H phase sensitive COSY spectrum of siroheme–cyanide in D_2O at pD 6.0 and 15 °C. The inset shows an enlargement of the region from 4.0 to 7.6 ppm. Also in this experiment, but not shown, are several distinct connectivities upfield of 4.5 ppm which were used to assign peaks (J,K,M,N,P,Q) in Table I. A total of 512 2K blocks were collected over 28 h, each consisting of 80 scans. A 12.5-μs 90° pulse was used with a sweep width of 72 000 Hz and a 1-s decoupler pulse to saturate the residual HDO signal. The data were processed using FTNMR software written by Dr. Dennis Hare.

Oxidized SiR-HP–CN Spectrum. Siegel et al. (1982) have shown that cyanide forms a one-to-one complex with SiR-HP. While the oxidized enzyme is relatively unreactive toward added ligands, rates of ligand binding are markedly increased if the enzyme heme becomes reduced. Thus, cyanide, at 1 mM, binds to oxidized SiR-HP with a $t_{1/2}$ = 8.5 h, while the binding of reduced SiR-HP occurs with a $t_{1/2}$ = ca. 1 s (Siegel et al., 1974; Janick et al., 1983). Formation of the reduced enzyme via photoreduction, addition of cyanide, and then reoxidation or, alternatively, prolonged incubation of enzyme with cyanide in SBE has been used to generate SiR-HP–CN samples, yielding identical results by NMR, optical, or EPR spectroscopies. The EPR and optical spectra agree with previously published results (Siegel et al., 1982; Janick & Siegel, 1983). Most samples used were generated by allowing a filter-sterilized solution of 300 μM SiR-HP and 5 mM KCN in pD 7.7 SB with a total volume of 2.5 mL to stand at room temperature in a 2 mm path length cuvette. The optical spectrum was recorded periodically until the alpha band position was at 581 nm. The reaction was typically complete in 16 h. The sample was then concentrated by Amicon filtration to a volume of 400–600 μL and transferred to an NMR tube.

The resulting samples generated the NMR spectra shown in Figures 5A and 6A. In contrast to results with the unligated enzyme (Kaufman et al., 1993b), Curie spin broadening is not apparent, and well-resolved spectra have been obtained at both 300 (Figure 5A) and 600 MHz (Figure 6A). Increased spectral resolution at higher field was obtained with the peaks at 11.88 to 11.51 being resolved into a triplet at higher field strength. The presence of the narrower line widths as compared to the unligated enzyme and prolonged spin–lattice

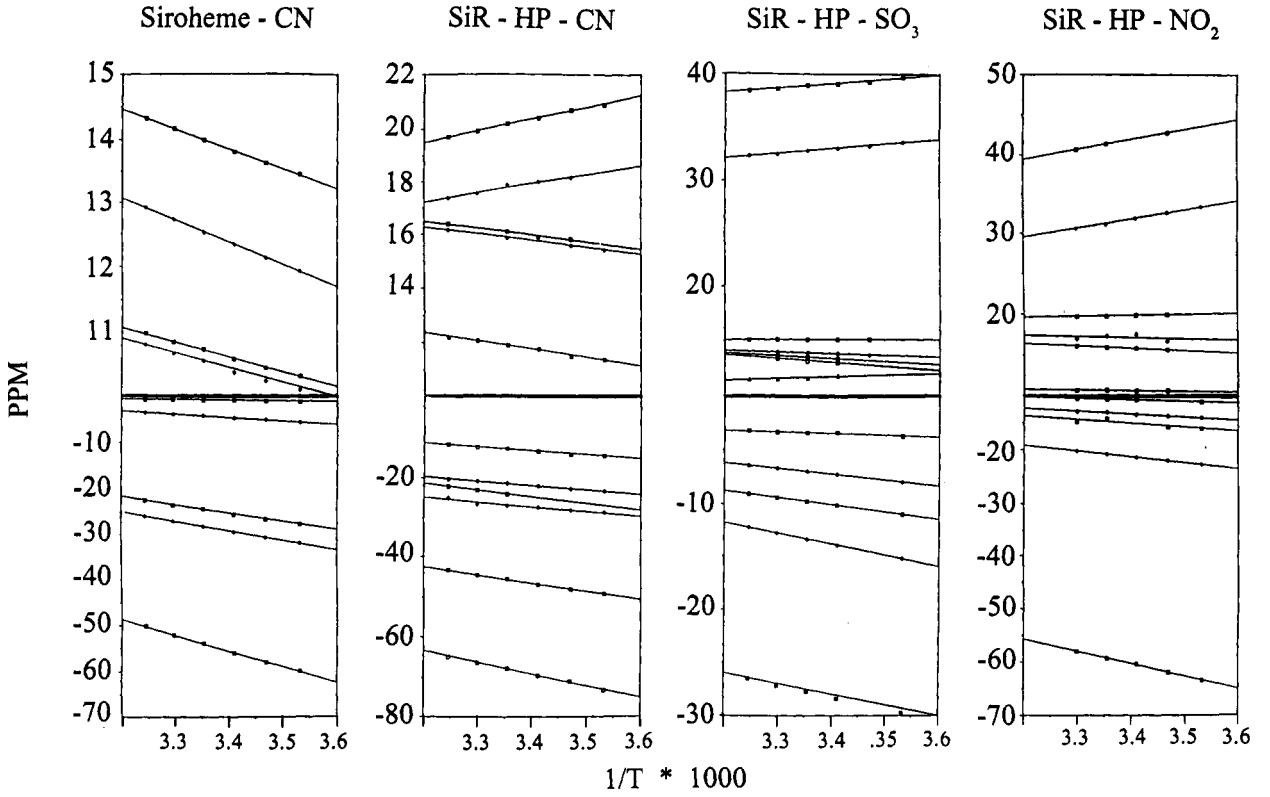

FIGURE 4: Temperature dependence of the paramagnetically shifted resonances in the NMR spectra of pD 6.0 siroheme–cyanide, SiR-HP–CN, SiR-HP–SO_3, and SiR-HP–NO_2. The NMR spectra were obtained at both 300 and 600 MHz as described in the legends of Figures 5 and 6 and calibrated vs internal DSS.

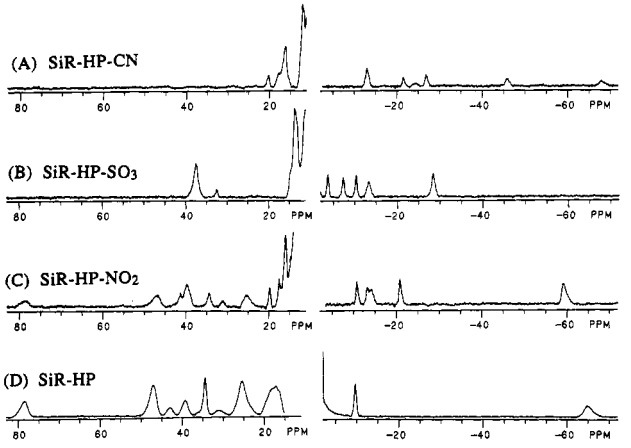

FIGURE 5: ^1H NMR (25 °C) spectra of oxidized unligated high-spin SiR-HP (D) and oxidized low-spin SiR-HP in 99.9% D_2O/50 mM KP_i (pD 7.7) bound to three different exogenous ligands [cyanide (A), sulfite (B), and nitrite (C)]. The samples were 1.4 mM in protein with a total volume of 450 μL. The NMR spectrum was obtained on a General Electric 300-MHz FT-NMR. An 8-μs observe pulse was used with a spectral width of 81 000 Hz over 16K data points in double-precision mode. The residual HDO signal was suppressed by a presaturation pulse of 200 ms from the decoupler. A total of 16 000 scans were collected over a period of 7 h. Line broadening of 15 Hz was applied to each spectrum. The baseline smoothing routine utilized resulted in some peak distortion.

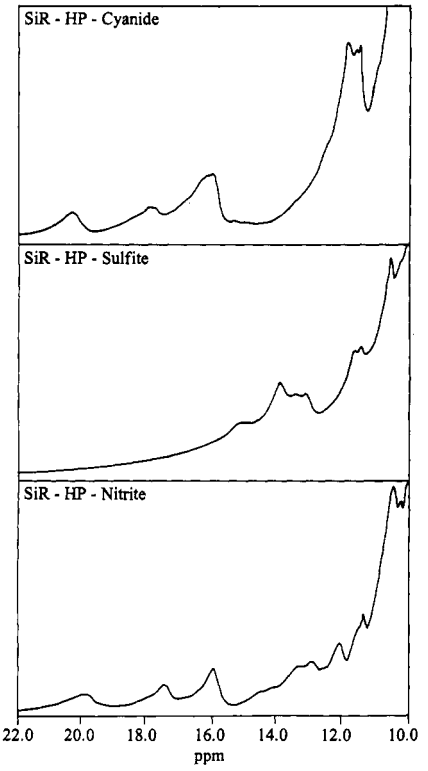

FIGURE 6: ^1H NMR at 600 MHz of SiR-HP ligated to cyanide (top), sulfite (middle), and nitrite (bottom) at 25 °C. Protein concentrations were 1.4 mM in pD 7.7 SB in a volume of 600 μL in a 5-mm NMR tube. An 8-μs observe pulse was used over a spectral width of 100 000 Hz.

relaxation times in SiR-HP–CN demonstrates that siroheme in SiR-HP–CN is ferric low spin (Satterlee, 1986; La Mar & Walker, 1979). No resonances arising from unligated enzyme are observed in the spectrum. The peak position, intensity, line-width, T_1, and temperature intercept data are summarized in Table II. Peaks were observed in the range 21 to –68 ppm. The observed spectrum is remarkably similar to that obtained for free siroheme–CN complex.

To distinguish iron–sulfur cysteine protons from those arising from the heme periphery, *E. coli* cells were grown on media containing DL-(*3,3,3′,3′-d*$_4$)-cystine; SiR-HP isolated from such cells contains deuterium attached to the β-carbon

Table II: ^{1}H NMR Data for SiR-HP Ligated to Exogenous Ligands

ppm[a]	intensity	T_1[b] (ms)	peak width	$1/T = 0$[c] (ppm)	assignment
		Cyanide Complex			
20.24	1	40 ± 2	160	5.5	
17.84	1	47 ± 5	175	6.0	
16.21	1	7.2 ± 0.3		25.0	β-CH_2-Cys
15.96	1	9.5 ± 0.4		24.3	β-CH_2-Cys
15.44	1			−80	
15.1	1				
11.88	1	19[d]			β-CH_2-Cys
11.66	1	19[d]			β-CH_2-Cys
11.51	1	19[d]			β-CH_2-Cys
−1.09	1 or 2				
−13.28	2	7.8 ± 0.4	325	19.3	β-CH_2-Cys
−21.69	1	11.9 ± 1.7	170	15.9	
−24.52	1	9.7 ± 2.1	330	29.8	
−27.05	1	11.6 ± 1.9	175	13.5	
−45.67	1	8.1 ± 2.3	340	24.5	
−67.23	1	5.2 ± 0.9	380	29.6	
		Sulfite Complex			
38.97	3	7.6 ± 0.5	365	25.3	
32.82	1	29.2 ± 2.7	140	17.6	
15.10	2	84.2 ± 5	128	14.9	
13.89	2	25.2 ± 0.7		19.4	β-CH_2-Cys
13.47	1	17.0 ± 0.6		22.4	β-CH_2-Cys
13.14	1	14.2 ± 0.4		25.0	β-CH_2-Cys
11.51	1	50 ± 0.8		6.32	
11.21	1	2–4	55		
10.54	1	64 ± 2.1	92	13.32	
−3.49	1	22.1 ± 1.4	113	2.11	
−7.06	1	19.9 ± 1.1	146	10.6	
−9.84	1	34 ± 0.8	162	14.1	
−13.45	1 or 2	8.1 ± 0.5	303	22.0	β-CH_2-Cys
−27.73	2	16.0 ± 0.9	286	9.90	
		Nitrite Complex			
41.3	2	26.0 ± 4.1	310	12.4	
31.15	1	5.3 ± 0.5	120	11.3	
19.76	1	69.1 ± 2.2		16.5	
17.32	1	59.5 ± 3.6		22.9	β-CH_2-Cys
15.921	2	14.7 ± 0.2		26.3	β-CH_2-Cys
10.575	1			13.0	
−10.808	1	50.8 ± 1.2	120	−1.0	
−13.248	1		140	4.7	
−14.411	1		110	9.6	β-CH_2-Cys
−20.919	1	29.8 ± 1.8	93	15.8	
−59.33	1	11.1 ± 0.3	325	19.0	

[a] Peak position for the sample at 25 °C in 1000 mM KP_i (pD 7.7). Sample referenced against internal DSS. Ligand concentrations were either 5 mM KCN, 100 mM KNO_2, or 50 mM K_2HSO_3. [b] Peak width calculated using best Lorentzian fit. [c] Extrapolated intercept at high temperature (Figure 4). [d] Unable to resolve due to peak overlap. Examination of T_1 data shows all three peaks have approximately the same T_1.

atom of all cysteine residues in the protein. The NMR spectrum of SiR-HP–CN incorporating β-CD_2-cystine is shown in Figure 7A. Examination of Figure 7A shows that six peaks (16.21, 15.96, 11.88, 11.66, 11.51, and −13.28 ppm) with a total intensity of approximately seven protons are missing in the β-CD_2-cysteine-labeled protein. In addition to the five single-intensity downfield-shifted peaks observed to arise from the cluster (not all peaks are resolved at 300 MHz, but they can be distinguished at 600 MHz), the peak at −13.3 ppm disappears in protein containing β-CD_2-cysteine. The presence of an upfield-shifted cluster cysteine resonance is strong evidence for continued heme-to-cluster coupling in solution for SiR-HP–CN. In addition, the presence of seven paramagnetically shifted cluster resonances means that cyanide cannot bind by displacing the bridging cysteine ligand; otherwise, no more than six hyperfine shifted β-CH_2-cysteine cluster resonances would be observed.

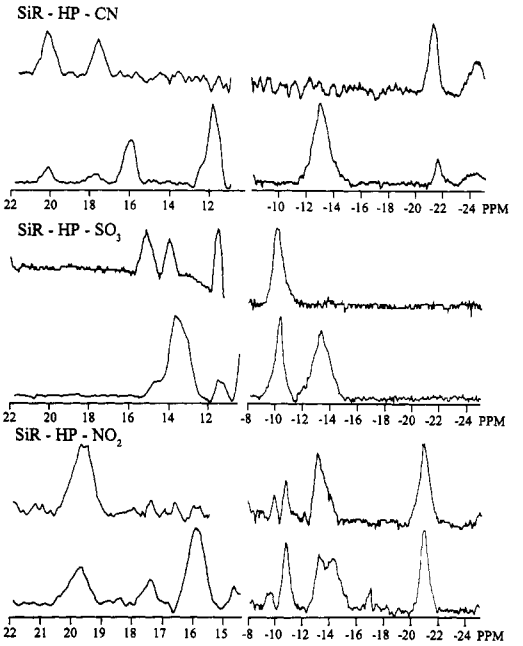

FIGURE 7: Upfield and downfield sections of ^{1}H NMR at 300 MHz of SiR-HP ligated to cyanide (1.3 mM; top), sulfite (950 μM; middle), and nitrite (1 mM; bottom), all obtained at 25 °C. The top spectrum for each complex shows enzyme prepared by incorporating β-CD_2-cystine. The spectra were all run with the conditions described in the legend of Figure 5.

Of the five downfield-shifted peaks determined by β-CD_2-cystine labeling to arise from cysteine-derived protons (Figure 7A; Table II), three peaks show anti-Curie temperature dependence, while two peaks are essentially temperature independent (Table II; Figure 4). The observation of anti-Curie temperature dependence for β-CH_2-cystine protons is characteristic of [4Fe-4S] clusters in the 2+ oxidation state (Poe et al., 1970; Phillips et al., 1974).

Reduction of SiR-HP–CN. To observe the NMR spectrum of the intermediate one-electron and fully reduced (two-electron) cyanide-ligated states, we subjected an anaerobic sample of SiR-HP (containing 10 mM EDTA-d_{12} and 150 μM of deazalumiflavin), in a sealed NMR tube, to photoreduction until the only form present was the fully reduced enzyme as determined by its NMR spectrum (Kaufman et al., 1993b). A small amount of 500 mM KCN (pD 7.7) was added anaerobically until the final KCN concentration was 10 mM, and the sample was then further photoreduced until no change could be detected by NMR spectroscopy.[3] The NMR spectrum obtained (Figure 8) consists of only four downfield-shifted proton resonances (65.8, 45.5, 20.7, and 15.3 ppm) and is taken to be that of SiR-HP–CN^{2-}. The two most downfield-shifted resonances have been shown to arise from cluster β-CH_2-cysteine protons (Figure 8) via β-CD_2-cystine incorporation. The sample was reoxidized by adding small amounts of either air or ferricyanide. In both cases, the identical intermediate NMR spectrum was observed before the spectrum of fully oxidized SiR-HP–CN was regained. This intermediate spectrum is identified as that of SiR-HP–CN^{1-} and consists of only two peaks at 15.5 and 13.8 ppm, with relative area 3:1, both of which have been shown by incorporation of β-CD_2-cystine to arise from cluster-derived protons. The spectra for SiR-HP–CN^{1-} and SiR-HP–CN^{2-}

[3] While the addition of KCN was nominally anaerobic, apparently not all oxygen was excluded. This is demonstrated by the presence of peaks arising from SiR-HP–CN^{1-}. Further photoreduction of the cyanide complex yielded pure SiR-HP–CN^{2-}.

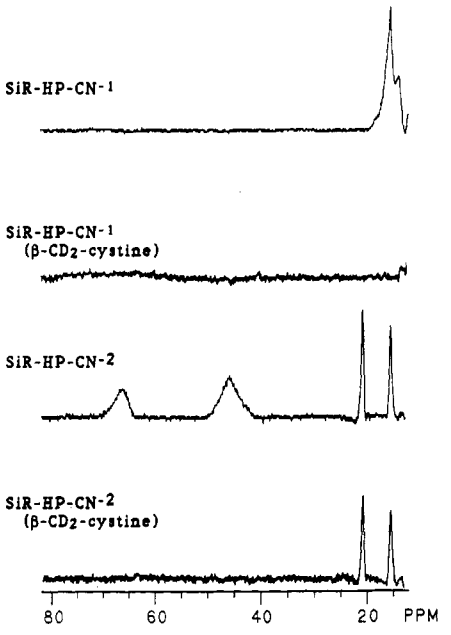

FIGURE 8: 300-MHz proton NMR spectra (25 °C) of the one- and two-electron reduced products of 1.2 mM SiR-HP–CN and SiR-HP–CN incorporating β-CD_2-cystine.

could also be generated by photoreducing a sample of SiR-HP–CN produced by prolonged incubation of heme protein with cyanide. Repeating the experiment in 90% H_2O yielded no exchangeable resonances with paramagnetic shifts.

SiR-HP–Sulfite Spectrum. The sulfite complex of SiR-HP was prepared by allowing a sterilized solution of 40 mM $NaHSO_3$ and 300 μM SiR-HP in pD 7.7 SB 99% D_2O with a total volume of 2.5 mL to stand at room temperature in a 2 mm path length cuvette. The optical spectrum was recorded periodically until the alpha band position was at 579 nm. The reaction was typically complete in 48 h. The sample was then concentrated by Amicon filtration to a volume of 400–600 μL and transferred to an NMR tube. Unlike the enzyme–cyanide complex, the SiR-HP–SO_3 complex was not formed by reacting reduced enzyme with sulfite because the partial reduction of sulfite would result. The 300-MHz spectrum of SiR-HP–SO_3 is given in Figure 5B. The improved spectral resolution available at higher field is illustrated in Figure 6B. The sample is clearly ferric $S = {}^1/_2$ in solution, despite the absence of a detectable EPR spectrum, as determined by narrow line widths, short T_1s (Table II), and absence of Curie spin broadening. The spectral range (39 to −28 ppm) shows larger downfield and smaller upfield shifts than does SiR-HP–CN. The temperature dependence data contain three single-intensity resonances with anti-Curie temperature dependence. These three resonances along with two other peaks, one of which is shifted upfield and has a relative intensity of two, can all be identified by β-CD_2-cystine labeling experiments as arising from cluster β-CH_2-cystine protons (Figure 7B). The presence of an upfield-shifted resonance is taken as evidence for the existence of coupling between the heme and cluster in sulfite-ligated enzyme in solution.

SiR-HP–Nitrite Spectrum. The nitrite complex of SiR-HP was prepared using methods identical to those described for preparing the sulfite complex except that the exogenous ligand was 100 mM KNO_2. The resulting SiR-HP–NO_2 NMR spectrum is shown in Figures 5C and 6C with the results summarized in Table II. While it was possible with the sulfite and cyanide complexes to generate NMR spectra with no detectable high-spin species present, incubation for 72 h with

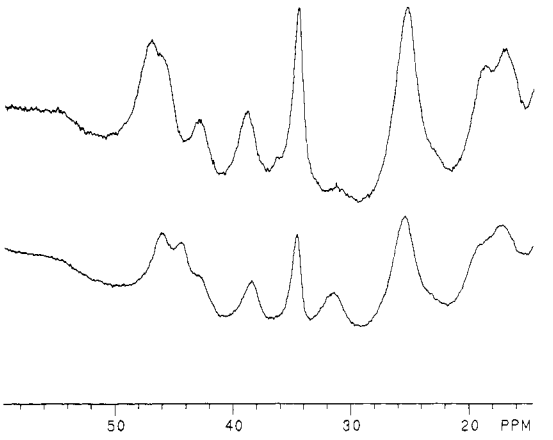

FIGURE 9: Section of the ^1H NMR spectra from 50 to 12.5 ppm of high-spin SiR-HP prior to (bottom) and after (top) the addition of sulfite to 40 mM.

nitrite failed to completely remove all peaks arising from the high-spin species (Figure 5C,D).[4] The spectrum of the nitrite complex is similar to that of the sulfite complex in that the two farthest downfield-shifted peaks occur at approximately 40 and 31 ppm. However, in contrast to the enzyme–sulfite NMR spectrum, peaks shifted as far as −59 ppm upfield are seen with SiR-HP–NO_2. The presence of narrowed peaks with reduced spectral range is taken as evidence that SiR-HP–NO_2 is ferric low spin. This is the first reported evidence that SiR-HP–NO_2 is low spin as it lacks a detectable EPR signal (Janick et al., 1983) and has not been investigated by magnetic susceptibility.[5] The temperature dependence data contain two resonances with anti-Curie temperature dependence, one of which possesses double-intensity (Figure 4). These resonances along with an upfield-shifted peak can all be identified by β-CD_2-cysteine labeling experiments as arising from cluster β-CH_2-cysteine protons (Figure 7). The presence of an upfield-shifted cluster resonance is taken as evidence for the existence of coupling between the heme and cluster in nitrite-ligated enzyme in solution. The presence of a similarly shifted upfield peak in the cyanide-, nitrite-, and sulfite-ligated enzyme suggests that all three ligands bind to SiR-HP via a similar mechanism.

Additional Ligand Binding. The addition to SiR-HP of each of the heme ligands used in the previous experiments was noted to cause immediate[6] and identical changes in the NMR spectrum of unligated SiR-HP (Figure 9). These spectral changes preceded conversion of SiR-HP to its final ligated spectrum. The immediate changes observed in the NMR spectrum correspond to a species that clearly has remained high spin ferric given the large line-width and spectral dispersion. Identical rapid changes were observed when chloride (40 mM), sulfite (40 mM), nitrite (100 mM), and nitrate (100 mM) were added to SiR-HP. This set of results

[4] All high-spin peaks present in Figure 5D are not detected in Figure 5C because of the limited concentration of high-spin species present (5–20% of 1.5 mM enzyme). In particular, high-spin peaks with short T_2s are not observed. For example, the broad resonance at −65 ppm in Figure 5D is not observed significantly above baseline in Figure 5C and should not be mistakenly associated with the resonance at −59 ppm in Figure 5C which is characteristic of the nitrite complex.

[5] Day et al. (1988) have shown that the EPR-silent nitrite complex of spinach nitrite reductase is also $S = {}^1/_2$ through magnetic susceptibility studies of that complex in the frozen state.

[6] The most rapidly obtained NMR spectrum was acquired over a period of 20 min following anion addition. At this time, all spectral changes that occur to the high-spin species upon anion addition were already complete.

initially led to the working hypothesis that the observed changes were due to ionic strength effects. However, no perturbation in the spectrum of SiR-HP could be observed in solution when the phosphate concentration was increased from 100 to 500 mM.

DISCUSSION

Low-Spin ($S = {}^1/_2$) Siroheme–CN. The macrocycle in siroheme is the equivalent of four electrons more reduced than that in porphyrins. Siroheme contains two partially saturated pyrroline rings in place of unsaturated pyrroles, and therefore the π orbital spin system is shorter and the ring current reduced from the porphyrin state. Abraham et al. (1987), using the dipole–dipole network model of ring currents, calculated a small (7%) decrease in the pyrrole ring currents and a pronounced (55%) decrease in the inner loop ring current on going from porphyrin to isobacteriochlorin. Spin transferred from metal d orbitals to a π ligand molecular orbital is delocalized over fewer bonds in isobacteriochlorins, and therefore the spin at a particular position would be greater than in the corresponding chlorin or porphyrin. The hyperfine shifts for low-spin ferric porphyrins are known to consist of small to moderate (3–6 ppm) upfield dipolar shifts due to magnetic anisotropy and larger contact shifts that are downfield for core methyls (Liccocia et al., 1989; La Mar & Walker, 1973). By the preceding argument, ferric low-spin isobacteriochlorins would be expected to have larger downfield shifts than the corresponding porphyrin or chlorin. The pyrrole portion of the siroheme macrocycle contains no methyl groups to use as a reference for spin density. However, the acetate and propionate groups that are present show minimal downfield shifts with the meso protons shifted significantly to higher field as compared to porphyrins or chlorins.

The macrocycle in siroheme is not a porphyrin but an isobacteriochlorin, and its symmetry is C_{2v}, not D_{4h}. Upon such a lowering of the iron site symmetry, the d orbital symmetries are reduced. The porphyrin a_{2u} orbital symmetry reduces to a_1, and the a_{1u} orbital symmetry reduces to a_2 (for clarity the D_{4h} symmetry labels of a_{1u} and a_{2u} are retained). This now allows these two orbitals to interact with the metal d orbitals which were previously symmetry-forbidden. No model low-spin isobacteriochlorins have as yet been studied. For the only low-spin chlorins studied, sulfmyoglobin (Chatfield et al., 1988) and pyropheophorbide (Licoccia et al., 1989), the pattern of contact shifts observed can be explained by π metal bonding involving the a_{1u} orbital. The a_{1u} orbital is the highest occupied molecular orbital in both chlorin and isobacteriochlorin (Chang et al., 1981). However, the spectral form observed for siroheme, with spin density located at the meso positions rather than the pyrrole, is better explained with the iron d orbitals interacting with the a_{2u} orbital.

A second possibility that must be considered in explaining the large upfield meso shifts in the cyanide complex is the role of the dipolar hyperfine shift. For low-spin ferric porphyrins, the hyperfine shifts are known to consist of only small to moderate (3–6 ppm) upfield dipolar shifts due to magnetic anisotropy (Licoccia et al., 1989). The in-plane iron to meso distance is almost identical for all four meso protons, which are all in the *xy* plane. However, while there is no crystallographically determined structure for siroheme, structures have been published for a four-coordinate (5,10,15,20-tetramethylisobacteriochlorinato)nickel(II) complex, which crystallizes as two saddle-shaped inequivalent molecules per unit cell (Suh et al., 1984) and a near planar [2,3,7,8-tetrahydro-5,10,15,20-tetraphenylporphinato(pyridine)zinc-(II)] (Barkigia et al., 1981). A tendency for bond-length alteration due to reduced aromaticity is apparent in these structures. The lack of planarity of an isobacteriochlorin means that the meso protons can have different angular dependence. The 5 and 20 meso protons are seen to be significantly below the plane of the macrocycle (dihedral angle 43°), while the 10 and 15 meso protons are essentially in the plane. The observed meso shift pattern of two pairs with almost identical average shift but with one pair widely separated (–5 and –58 ppm) can be explained as an angular effect on the dipolar shift. The two meson protons in the macrocycle plane are located on a nodal plane and experience little dipolar shift, while the 5 and 20 meso protons are located in lobes of opposite sign but experience shifts of approximately equal magnitude.

A third alternative that must be considered is the formation of π–π aggregates on the observed chemical shift. Some low-spin bis(cyanide) porphyrins have been shown to aggregate (Viscio et al., 1978) at low temperature in a concentration-dependent manner. This aggregation could place a meso proton close to the iron of its neighbor, allowing it to sense significant dipolar shift. This hypothesis is also supported by the anti-Curie temperature dependence of peaks A–D. However, the SiR-HP–CN complex, where the heme cannot be aggregated, shows a remarkably similar spectrum to that of siroheme–CN. While this does not rule out aggregation as the cause of the observed anti-Curie temperature dependence, it suggests that siroheme–CN can still be used as a model for studying the enzyme. Another possible explanation is that the hydroporphyrin siroheme exists in more than one conformation in solution and the equilibrium mixture is temperature-dependent.

Analysis of Observed Shifts in SiR-HP–CN. The first low-spin complex of SiR-HP chosen for study was the cyanide complex. Extensive and in-depth NMR studies exist in the literature on ferric–porphyrin–cyanide complexes (Satterlee, 1986; La Mar & Walker, 1979; Shulman et al., 1971), and recently reports have appeared for ferric–chlorin–cyanide complexes (Keating et al., 1992; Liccocia et al., 1989). The NMR spectrum obtained for SiR-HP–CN (Figures 5A and 6A) differs markedly from those observed for protein–chlorin complexes with cyanide. The observation of small downfield and large upfield shifts for the protein is consistent with spin density being transferred into the a_{2u} MO rather than the a_{1u} MO. Despite containing only a single bound cyanide [while the free heme is believed to be the bis(cyanide) complex] and an iron–sulfur cluster, SiR-HP–CN exhibits a NMR spectrum which is very similar to that of free siroheme–CN.

In addition to a $S = {}^1/_2$ ferric siroheme, SiR-HP–CN also contains a low-potential [4Fe-4S] cluster in the 2+ oxidation state (Siegel et al., 1973, 1982; Janick et al., 1982). Paramagnetic NMR spectroscopy is useful not only for examining heme-containing proteins but also for investigating the structure and electronic properties of iron–sulfur proteins. The observed pattern of hyperfine shifted resonances along with their temperature dependence can provide detailed information on bonding and magnetic interactions within a cluster. While [4Fe-4S] clusters in the 2+ oxidation state are nominally diamagnetic, hyperfine shifted resonances are observed due to the population of excited states at room temperature. In oxidized bacterial-type ferredoxins, four to eight hyperfine shifted resonances arising from cluster-coordinating β-CH_2-cysteines and possessing anti-Curie temperature dependence are observed in the range 8–20 ppm (Packer et al., 1977; Skjeldal et al., 1989; Poe et al., 1970).

Using β-CD_2-cystine-labeled enzyme, six resonances with a total integrated intensity of seven protons have been identified as arising from cluster-derived cysteine protons. The observed resonances differ from the typical NMR pattern for $[4Fe\text{-}4S]^{2+}$ clusters in two respects: both Curie and anti-Curie temperature dependencies are observed, and an upfield-shifted β-CH_2-cysteine resonance of intensity two is present. These differences can rationally be attributed to the presence of a $S = {}^1/_2$ ferric siroheme.

The presence of a paramagnetic heme can affect the cluster via two possible mechanisms. Dipolar (pseudocontact) interactions can result via a through-space effect which is determined by the specific geometry and distance from the siroheme metal (distance to the third power). Low-spin ferric hemes exhibit highly anisotropic g tensors; therefore, they can yield significant dipolar shifts (Satterlee, 1986). A second mechanism for producing hyperfine shifts is via contact or scalar coupling and is due to through-bond electron transfer. For such a mechanism to occur, there must exist a bridging ligand between heme and cluster. A bridge has been shown to exist between heme and cluster in the unligated enzyme in solution (Kaufman et al., 1993b) and in the cyanide complex in the frozen state (Christner et al., 1983b). If the observed hyperfine cluster shifts in SiR-HP–CN cannot be explained solely as a dipolar effect, a contact interaction would be necessary which requires a bridge between heme and cluster. Therefore, the relative size of the dipolar shift must be determined and compared to the observed hyperfine shifts in SiR-HP–CN.

The largest observed cluster-derived hyperfine shift belongs to the β-CH_2-cystine resonance at –13.28 ppm. Its position, in the absence of the heme, would be expected to be between 8 and 16 ppm. By using the midpoint of these two values as a reference, we can calculate that the peak is isotropically shifted approximately –25 ppm. Unfortunately, the exact theoretical calculation of a dipolar shift is not currently possible even if the proton-to-heme iron distance and geometric factors are known (Satterlee, 1986; Shulman et al., 1971). In addition, siroheme is a nonplanar isobacteriochlorin; therefore, simplified models used for porphyrins may not be applicable. Experimental techniques used to separate the dipolar and contact shift components, on the basis of their differing temperature dependencies, are not applicable here because of cluster effects (La Mar & Walker, 1979; Satterlee, 1986). However, two lines of evidence suggest that the magnitude of the dipolar contribution is small. The size of the dipolar shifts observed in low-spin ferric porphyrins is dependent on the magnitude of the magnetic anisotropy present. This can be estimated on the basis of EPR-derived g values (La Mar & Walker, 1979). On the basis of the known EPR g values for SiR-HP–CN (g = 2.39, 2.33, and 1.67; Janick et al., 1983), a smaller degree of magnetic anisotropy can be expected as compared to reports in the literature (La Mar & Walker, 1979). Second, examination of the heme protein literature indicates virtually no large upfield paramagnetic shifts for non-heme ligated amino acids. These observations suggest that a purely dipolar effect is unlikely to cause the large upfield shift observed. More conclusive arguments await either improved theoretical calculations or the generation and NMR characterization of a linked cluster to heme model complex.

The identity of the upfield-shifted cluster resonance is currently unknown. In the unligated high-spin complex, a single cluster-derived peak with intensity two was seen to be shifted upfield (–63.3 ppm; Kaufman et al., 1993b). This peak was not assigned to the bridging cysteine ligand. Mössbauer studies of SiR-HP in the frozen state (Christner et al., 1983a) show that the heme Fe–cluster coupling decreases in intensity by a factor of 3 on cyanide binding. Using the model previously proposed (Kaufman et al., 1993b), which relates the size of the spin density transferred from heme to cluster to the magnitude of the observed cluster hyperfine shift, the Mössbauer data would predict a decrease in magnitude by approximately 3 for the cluster proton isotropic shifts on going from high- to low-spin siroheme. This prediction is consistent with the observed decrease in the isotropic shift of the upfield resonance (from approximately –75 to –25 ppm) on going from the high-spin to the low-spin complex. While this makes it tempting to assign the sole upfield-shifted peak in the low-spin complexes to this same cysteine resonance, several electronic and structural changes occur upon the conversion of a high-spin ($S = {}^5/_2$) siroheme to a low-spin ($S = {}^1/_2$) complex which make this analogy uncertain. Structurally, these changes involve the conversion from a pentacoordinate "out of plane" conformer for the Fe(III) heme atom to a hexacoordinate "in-plane" structure upon addition of the axial CN^- ligand. Comparative X-ray crystallography studies on Fe(III) protoporphyrin IX complexes show that the movement of the Fe atom relative to the porphyrin plane can be as large as 0.50 Å (Countryman et al., 1969). This change would be expected to increase the length of the Fe–Cys sulfur bond, hence weakening the magnetic interaction. Such large movements of the bridging ligand would also be expected to change the geometry of the cluster, which would change the angular dependencies of the cluster cysteine shifts and make direct comparison impossible.

However, a more important consideration in this case is the change in electronic orbital population on going from high to low spin. For the high-spin pentacoordinate center, unpaired electron density resides in iron orbitals of both σ ($d_{x^2-y^2}$;d_{z^2}) and π (d_{xy};d_{xz};d_{yz}) symmetry; these can interact with either σ or π orbitals of the cysteine bridge in fostering exchange interaction with the cluster. In the low-spin case, unpaired spin density on the heme iron is confined to an orbital of only π symmetry (mainly d_{yz}) relative to the cysteine bridge so that any σ exchange would have to occur via a spin polarization mechanism at the iron center. The angular dependence of σ and π interactions differ, and their relative contributions are unknown.

Lastly, a corresponding decrease in the downfield-shifted resonances on going from high to low spin is not observed. All observed downfield cluster shifts are in the range expected for an isolated $[4Fe\text{-}4S]^{2+}$ cluster. They would be expected to have much larger hyperfine shifts on the basis of our previously proposed model (Kaufman et al., 1993b), if the heme-to-cluster decreased coupling was the sole explanation of the lessened shifts.

A second possible identity to the upfield-shifted resonance that must be considered is that it belongs to the cysteine bridging between heme and cluster. While there are no model low-spin isobacteriochlorins ligated to cysteine available for comparison, papers exist in the literature of NMR studies on low-spin ferric porphyrin complexes ligated to both cysteine and methionine. The presence of an axial cysteine ligand to the heme iron has been demonstrated for the enzymes chloroperoxidase and cytochrome P-450. The NMR spectrum of the cyanide complex of cytochrome P-450 shows no upfield-shifted resonances and the chemical shift of the coordinating β-CH_2-cysteine is not assigned (Keller et al., 1972). In chloroperoxidase, two peaks at 38.6 and –20.6 ppm have been

assigned to the coordinating cysteine because of their short T_1s (Dugad et al., 1992).

The γ-CH_2 protons of a coordinating methionine are in comparable positions to the β-CH_2 protons in cysteine and can also be used as a model to estimate the chemical shift.[7] In a series of low-spin cytochrome complexes the γ-CH_2 protons of the coordinating methionines were observed to occur in the range −8 to −32 ppm (Satterlee, 1986). An additional factor present in SiR-HP, however, which is not included in these models is the effect of the cluster. None of the other cluster-derived cysteine peaks show large perturbation from the chemical shift range expected for an isolated ferredoxin with net contact shifts approximately 10 ppm downfield (Packer et al., 1977; Skjeldal et al., 1989; Poe et al., 1970). Allowing for this tendency of the cluster to shift cysteine resonances modestly downfield, the upfield-shifted peak in SiR-HP–CN still possesses a chemical shift consistent with assignment of this peak to the siroheme coordinating cysteine.

Numerous studies (Siegel et al., 1973, 1982; Janick et al., 1982; Han et al., 1989; Christner et al., 1981, 1983a,b; Madden et al., 1989) have shown that SiR-HP–CN^{1-} contains a ferrous $S = 0$ heme and a [4Fe-4S] cluster in the 2+ ($S = 0$) oxidation state, while SiR-HP–CN^{2-} contains a ferrous $S = 0$ heme and a [4Fe-4S] cluster in the 1+ ($S = {}^1/_2$) oxidation state. Low-spin ferrous siroheme has no unpaired electrons ($S = 0$), and the $S = 1$ excited states are at least a factor 100 times higher in energy than (Gutlich, 1981) those of the [4Fe-4S] cluster. Hence, population of the excited states at room temperature should be limited solely to the cluster. This means that only cluster resonances would be expected to show proton hyperfine shifts. Therefore, all of the observed proton resonances for SiR-HP–CN^{-1} and SiR-HP–CN^{-2} would be expected to arise from cluster-derived protons, particularly from the coordinating cysteine residues.[8] The NMR spectrum of these two species can be used to determine the magnetic properties of the cluster in "isolation" from the heme. While the observed range of chemical shifts is identical to those reported for ferredoxin [4Fe-4S] clusters in the 2+ (Skjeldal et al., 1989) and 3+ (Phillips et al., 1974; Poe et al., 1970) oxidation states, the total number of observed β-CH_2-cysteine peaks is less than expected. In SiR-HP–CN^{-1}, two peaks are observed with relative intensity 3:1. On the basis of the ratio of integrated intensities this represents either four or eight of the eight protons expected. SiR-HP–CN^{-2} shows only two of the eight expected β-CH_2-cysteine protons. The remaining six protons likely possess short T_1s and T_2s (particularly given the higher molecular weight of SiR-HP compared to ferredoxins), and therefore the extensively broadened resonances are not observed.

SiR-HP Ligated to Sulfite and Nitrite. Sulfite and nitrite are substrates which siroheme-containing enzymes reduce to sulfide and ammonia, respectively (Krueger & Siegel, 1982). Sulfite- or nitrite-ligated siroheme enzyme complexes fail to generate quantifiable EPR signals. Magnetic susceptibility measurements have shown both SiR-HP–SO_3 and the nitrite complex of nitrite reductase from spinach to be $S = {}^1/_2$ (Day et al., 1988). This conclusion is readily confirmed for SiR-HP–SO_3 and extended to SiR-HP–NO_2 by NMR spectroscopy which shows narrowed line widths and prolonged T_1s in these enzyme complexes. Several similarities and differences exist between the NMR spectra of the two species.

The major difference between the two species is the inability to solely generate the low-spin complex by prolonged incubation of SiR-HP with nitrite. This is consistent with other studies (Young & Siegel, 1988) which have estimated the K_d of nitrite binding to SiR-HP to be 5 mM; despite prolonged incubation at high concentrations of nitrite, approximately 5–20% of SiR-HP remained unligated. Sulfite has a measured K_d of 7 μM, while cyanide binding is essentially irreversible (Janick et al., 1983).

The nitrite complex also possesses a peak shifted farther upfield (−59.3 ppm) as compared to the sulfite complex (−27.8 ppm). This upfield-shifted peak is not a residual high-spin peak, nor is it derived from the cluster. A similarly shifted upfield peak is seen in the cyanide complex (−67.2 ppm).

All three complexes studied (cyanide, nitrite, and sulfite) have a single upfield-shifted β-CH_2-cysteine resonance present at almost identical chemical shifts (−13.3, −13.5, and −14.4 ppm). The presence of an upfield-shifted cysteine resonance in the sulfite and nitrite SiR-HP complexes can be interpreted, as discussed previously, as evidence for the existence of a bridging ligand. Both the cyanide– and sulfite–enzyme complex resonances have been assigned to at least twice unit intensity, with the nitrite complex resonance having only single intensity.[9] What is remarkable about this upfield peak is how stable its position is in all three complexes. If this peak is assigned to the bridging ligand, it would be expected to be more sensitive to the nature of the distal ligand which is changed in each complex. If this peak does not belong to the bridging ligand, its geometry and spin density may not change as the heme ligand changes. The cysteine resonances observed downfield, however, do change position as the heme ligand is changed (Figure 7). In addition, they show much smaller isotropic shifts as compared to the upfield-shifted resonance. The model proposed for the high-spin complex (Kaufman et al., 1993b) divides the cluster irons into two equivalent iron pairs with equal and opposite spin density. As the size of the predicted isotropic shift depends on the magnitude and sign of the spin density, equal and opposite hyperfine shifts would be expected. The unligated high-spin complex shows cysteine resonances with large downfield and smaller upfield shifts. The differing magnitude of the isotropic shifts may be a result of differing angular dependencies as the size of the isotropic shift also has a dihedral angular dependence. The low-spin complexes of SiR-HP examined in this work show large upfield and small downfield shifts. Again, as in the high-spin enzyme, differing angular dependencies can be invoked to explain the inequality in the magnitude of the isotropic shifts.

The only previously published NMR spectrum for a low-spin siroheme–[4Fe-4S] cluster (Cowan & Sola, 1990) is for the assimilatory sulfite reductase from *Desulfovibrio vulgaris* (Hildenborough). This enzyme was postulated by these authors to consist of a [4Fe-4S] cluster coupled to siroheme via a bridging sulfide ligand with a histidine as the distal

[7] The γ-CH_2 protons of coordinating methionine can also be used to help model the unligated high-spin complex. In earlier studies, we demonstrated that a peak at 55 ppm was derived from the bridging cysteine residue (Kaufman et al., 1993b). The size of the high-spin contact shift for γ-CH_2 methionine protons in ferricytochrome b_{562} at 45 °C has been estimated at approximately 470 ppm (Wu et al., 1991a). A downfield contact shift of this type in SiR-HP will be modulated by an angular dependence term plus an additional upfield contact shift derived from the cluster (Kaufman et al., 1993b). The observed net chemical shift is consistent with all of these influences.

[8] This includes protons derived from both the β-CH_2 and α-CH positions of cluster coordinating cysteines. In this work, we deuterate protons on only the β-carbon.

[9] These intensities are the best estimates available because in all three complexes, particularly the nitrite, no single area value as unit intensity led to integer proton intensities. The presence of multiple species with only slightly differing NMR spectra cannot be excluded.

ligand. NMR resonances were observed in the range from 92.5 downfield to −13.3 upfield. This complex is not directly comparable to those presented here due to the differing heme ligands. However, a resonance is observed at −13.3 ppm in the *D. vulgaris* SiR. This is the location, in all three low-spin SiR-HP complexes presented here, that a β-CH_2-cysteine-derived resonance occurs, which is unique to this coupled system.

Additional Ligand Binding. Addition of high concentrations of a variety of anions to SiR-HP was found to cause immediate and identical changes in the NMR spectrum of unligated SiR-HP (Figure 9). These changes were observed prior to ligand binding at the heme iron; the species present has clearly remained high spin ferric, given the large linewidth and spectral dispersion. Similar changes were observed with chloride (40 mM), sulfite (40 mM), nitrite (100 mM), and nitrate (100 mM). However, no perturbation in the NMR spectrum was found when the phosphate concentration was increased from 100 to 500 mM. We interpret the latter result as ruling out an ionic strength effect as the sole cause of these spectral changes. The most likely hypothesis to explain these changes is the presence of a secondary anion binding site in SiR-HP. Occupancy of this site may cause a conformational change at the heme, resulting in the altered spectrum.

Summary. In this work we report the first published spectrum of an isolated low-spin ferric isobacteriochlorin (siroheme–CN). The NMR spectrum of isolated siroheme–CN is consistent with spin density being transferred into the a_{2u} molecular orbital, an interaction which is symmetry-forbidden in porphyrins. The NMR spectra for the cyanide-, sulfite-, and nitrite-ligated sulfite reductase heme protein subunit are also reported. All ligated states studied possess an almost identically shifted upfield cluster resonance whose presence can be interpreted as evidence that covalent coupling exists between siroheme and cluster in solution. Data are also presented that suggest the existence of a secondary anion binding site, the occupancy of which perturbs the oxidized SiR-HP NMR spectrum, where binding occurs at a rate much faster than that of ligand binding to enzyme ferriheme.

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