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Resonance Raman Studies of *Escherichia coli* Sulfite Reductase Hemoprotein. 2. Fe_4S_4 Cluster Vibrational Modes†

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ABSTRACT: Resonance Raman (RR) spectra from the hemoprotein subunit of *Escherichia coli* sulfite reductase (SiR-HP) are examined in the low-frequency (200–500 cm^{-1}) region where Fe–S stretching modes are expected. In spectra obtained with excitation in the siroheme Soret or Q bands, this region is dominated by siroheme modes. Modes assignable to the Fe_4S_4 cluster are selectively enhanced, however, with excitation at 488.0 or 457.9 nm. The assignments are confirmed by observation of the expected frequency shifts in SiR-HP extracted from *E. coli* grown on ^{34}S-labeled sulfate. The mode frequencies and isotopic shifts resemble those seen in RR spectra of other Fe_4S_4 proteins and analogues, but the breathing mode of the cluster at 342 cm^{-1} is higher than that observed in the other species. Spectra of various ligand complexes of SiR-HP reveal only slight sensitivity of the cluster terminal ligand modes to the presence of exogenous heme ligands, at variance with a model of ligand binding in a bridged mode between heme and cluster. Close examination of RR spectra obtained with siroheme Soret-band excitation reveals additional ^{34}S-sensitive features at 352 and 393 cm^{-1}. These may be attributed to a bridging thiolate ligand.

The active site of *Escherichia coli* sulfite reductase hemoprotein (SiR-HP) comprises an Fe_4S_4 cluster exchange coupled to a siroheme prosthetic group (Christner et al., 1981; Janick & Siegel, 1982, 1983; Siegel et al., 1982). A model of the active site has been proposed (Christner et al., 1984) in which one cluster Fe is covalently bridged to the siroheme Fe by an S atom of a cysteinyl ligand. The X-ray crystal structure (McRee et al., 1986) is consistent with this model; it shows the cluster to be closely apposed to the heme and reveals that one of the Fe_4S_4 cubane sulfur atoms is in van der Waals contact with the siroheme periphery. At the present level of resolution, however, the putative bridging thiolate itself is not clearly discernible.

Structurally, the Fe_4S_4 core in SiR-HP appears to conform closely to that observed in "typical" four-iron clusters. The electron density map of the cluster region in SiR-HP is well fit by an idealized Fe_4S_4 model derived from crystallographic data from previously characterized clusters (McRee et al., 1986). The SiR-HP cluster has been studied by magnetic spectroscopy in a variety of derivatives of the enzyme and does not appear to deviate significantly from representative Fe_4S_4 clusters in its internal electronic structure. Thus, the Mössbauer parameters, ΔE_Q and δ, of the cluster irons (Christner et al., 1981; Janick & Siegel, 1982, 1983) in both the oxidized (2+) and reduced (1+) states are essentially the same as those observed in other well-characterized Fe_4S_4 proteins such as *Bacillus stearothermophilus* ferredoxin (Münck & Kent, 1987). Upon reduction of CN^-- or CO-ligated SiR-HP the expected $g = 1.94$ EPR signal typical of Fe_4S_4 clusters in the 1+ state is observed (Janick & Siegel, 1982, 1983). On the other hand, the Mössbauer measurements of the magnetic coupling between the siroheme and the cluster irons in SiR-HP are markedly sensitive to the electronic state of the siroheme, and novel EPR signals are observed for the

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reduced cluster when the siroheme iron is in a paramagnetic state, effects that provide direct evidence of electronic coupling between the siroheme and cluster (Christner et al., 1981, 1983, 1984). The amino acid sequence at the putative cluster-binding site of SiR-HP also differs significantly from the cluster-binding sequences observed in any previously investigated class of four-iron proteins (Ostrowski et al., 1987).

In the preceding paper in this series (Han et al., 1989a) we reported resonance Raman (RR) spectra of the siroheme moiety of SiR-HP. Because of the number and intensity of the heme-associated bands in the low-frequency region of the RR spectrum, identification of vibrational bands arising from the cluster initially proved difficult. We have now succeeded in identifying modes associated with the Fe_4S_4 cluster with excitation at 488.0 or 457.9 nm and have confirmed the assignment of these modes by recording the spectra of protein extracted from *E. coli* grown on ^{34}S-labeled sulfate. We have also investigated the effect of the ligation and reduction state of the siroheme iron on the cluster spectrum. Direct evidence of a heme–cluster connection has been sought in the form of a RR band assignable, via its ^{34}S shift, to the stretching mode of the putative bridging thiolate ligand; although no prominent band of this character could be found, weak ^{34}S-sensitive features that might be assigned to the symmetric and antisymmetric Fe–S–Fe stretch of a siroheme–cluster bridge have been discerned by using excitation into siroheme absorption bands.

Experimental Procedures

Preparation of ^{34}S-Labled Sulfate. Sodium [^{34}S]bisulfate was prepared from elemental ^{34}S (93.65 atom %, Monsanto Research Corp., Miamisburg, OH) by oxidation in Br_2/HNO_3, followed by addition of NaCl and repeated dissolution and evaporation of the residue in concentrated HCl (Furman, 1961). The yield was quantitative as determined by a turbidimetric method (Jackson & McCandless, 1978).

Growth of ^{34}S-Labeled E. coli. Cells of *E. coli* B (ATCC 11303) were grown at 37 °C in a 200-L fermenter by using the minimal medium described by Siegel et al. (1973), except that the corresponding chloride salts were substituted for the sulfate salts in all cases. Sodium [^{34}S]bisulfate was added to a final concentration of 1 mM. The medium (130 L) was inoculated with cultures pregrown on [^{34}S]sulfate medium. The cells were harvested in early stationary phase (yield, 0.85 kg) and stored at −15 °C.

Purification of Sulfite Reductase Hemoprotein. Sulfite reductase holoenzyme subunit was purified and dissociated as previously described (Siegel et al., 1973). The hemoprotein fraction was separated by DEAE-cellulose chromatography and was further purified as previously described (Han et al., 1989a). During the preparation of the ^{34}S-labeled protein, the fraction that remained bound to the DEAE-cellulose column, consisting of the flavoprotein subunit of sulfite reductase together with some undissociated holoenzyme, was eluted with 1 M potassium phosphate (pH 7.7) containing 4 M urea and was retained for use in GC–MS isotopic analysis. The purified ^{34}S-labeled hemoprotein sample (45 nmol) had an A_{280}/A_{386} ratio of 1.72 and optical and EPR spectra identical with those of unlabeled SiR-HP.

Determination of $^{34}S/^{32}S$ Isotopic Ratio. A dialyzed sample of the DEAE eluate that had been recovered for mass spectral analysis was reduced and methylated by using a modification of a standard procedure (Crestfield et al., 1963) (methyl iodide was used in place of iodoacetate). The dialyzed, lyophilized residue was hydrolyzed in vacuo (6 N HCl, 125 °C, 18 h), and the resulting amino acid hydrochloride salts were converted to their *N*-heptafluorobutyryl methyl esters (White, 1981). The extent of ^{34}S labeling in cysteine (as *S*-methylcysteine) and methionine was determined by means of selected ion monitoring at the M^+ peaks using a VG TRIO-2 GC-Quadrupole MS (VG Masslab, Ltd., Altrincham, U.K.). The $^{34}S/^{32}S$ ratio in the labeled protein was 14.1 for cysteine and 18.3 for methionine.

Sample Preparation for RR Spectroscopy. Samples of natural abundance and ^{34}S-labeled SiR-HP in 0.1 M potassium phosphate, pH 7.7, containing 100 mM EDTA (standard buffer) were concentrated by ultrafiltration. Heme concentration was determined optically by using $\epsilon_{591} = 18\,100\ cm^{-1}$ (Siegel et al., 1982). Due to the slow rate of reaction of oxidized SiR-HP with exogenous ligands, preparation of complexes of SiR-HP with CN^-, CO, or NO requires prior reduction of the enzyme. Solutions of Cr^{II}(EDTA), prepared as previously described (Han et al., 1989a), were used as the reductant. Details of sample preparation are provided in the figure legends.

Raman Spectroscopy. For Raman spectroscopy the matched samples were held frozen at 77 K in the sample cup of a low-temperature Raman cell. The instrumental arrangement has been previously described (Han et al., 1989a).

Results and Discussion

Identification of Cluster Modes. As we have previously demonstrated (Han et al., 1989a), laser excitation in the major absorption bands of sulfite reductase hemoprotein (SiR-HP) produces strong resonance Raman scattering from the siroheme. The siroheme RR spectrum includes numerous bands in the low-frequency region, where vibrational modes of the iron–sulfur cluster are expected (Spiro et al., 1988). With excitation at 457.9 or 488.0 nm, however, enhancement of the heme-associated modes is greatly attenuated. These wavelengths lie in the trough of the siroheme absorption spectrum, between the Soret and Q bands, but they are near the maximum of excitation profiles determined for Fe_4S_4 clusters (Czernuszewicz et al., 1987). The spectra of oxidized SiR-HP (SiR-HP^0) obtained at 457.9 and 488.0 nm, shown in Figure 1, contain a series of bands between 300 and 400 cm^{-1}, which resemble those exhibited by Fe_4S_4 proteins and analogues, and are distinctly different from the siroheme bands found in this region in the RR spectra obtained with Soret or Q-band excitation. The siroheme contribution at 457.9 or 488.0 nm is negligible, as shown by the absence of the strong 323-cm^{-1} band seen at 406.7 nm or the 410-cm^{-1} band seen at 568.2 nm.

The spectra shown in Figure 2 (lower panel) establish that the bands observed with 457.9- or 488.0-nm excitation are indeed iron–sulfur modes, since they show distinctive frequency shifts in SiR-HP extracted from *E. coli* grown on ^{34}S-labeled sulfate. The strongest band, at 342 cm^{-1} in the unlabeled protein, shifts downward by 9 cm^{-1} upon isotopic substitution; two other, weaker bands at 356 and 371 cm^{-1} shift downward by 5 cm^{-1}. A fourth, much weaker isotope-sensitive band may be present at around 390 cm^{-1} in the unlabeled protein. In Figure 2 (upper panel), the corresponding spectra of the oxidized cyanide complex of SiR-HP are displayed. The position of the strongest band is slightly higher than in the free, oxidized enzyme (344 vs 342 cm^{-1}), as is also the case for one of the two weaker bands (360 vs 356 cm^{-1}). For all three bands, downshifts are observed in the ^{34}S-labeled sample that are identical with those observed in the free enzyme. In addition, the broad, weak isotope-sensitive band centered at around 390 cm^{-1} is more clearly discerned; it appears to be composed of two components, both of which shift downward by $\sim$7 cm^{-1} in the ^{34}S-labeled sample.

Table I: RR Band (cm^{-1}) Correlation for the SiR-HP Fe_4S_4 Cluster with Various Fe_4S_4 Models

symmetry				*C. pasteurianum*			
D_{2d}	T_d	mode character	$[Fe_4S_4](-SCH_2Ph)_4$[a]	ferredoxin[b]	aconitase as isolated[c]	SiR-HP^0	SiR-HP^0–CN$^-$
A_1	A_1	ν(Fe–S_b)	333 (9)[d]	338	342	342 (9)[d]	344 (9)[d]
B_2				351	358	356 (5)	360 (5)
	T_2	ν(Fe–S_t)	358 (7)				
E				363	371	371 (5)	371 (5)
B_2	T_2	ν(Fe–S_b)		380			389 (7)
			384 (6)		400	~390	
A_1	A_1	ν(Fe–S_t)		395			396 (7)

[a] Czernuszewicz et al. (1987). Data for frozen solution in DMF. Benzyl cube crystals show splittings due to the $T_d \rightarrow D_{2d}$ symmetry lowering. [b] Czernuszewicz et al. (1987). Frozen solution at 77 K. [c] Johnson et al. (1983). Contains three-iron cluster, shown for comparison only. Symmetry assignments do not apply. [d] Numbers in parentheses are ^{34}S isotope shifts for doubly labeled (^{34}S at both bridging and terminal position) clusters.

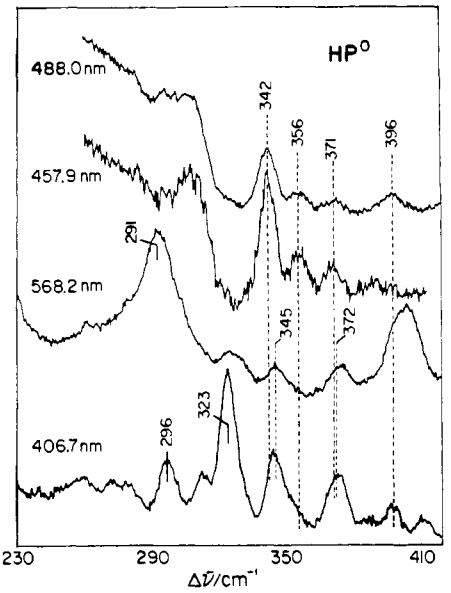

FIGURE 1: RR spectra of HP^0 (811 μM) in standard buffer at 77 K using various excitation wavelengths. The cluster modes are selectively enhanced with 457.9- or 488.0-nm excitation, while the siroheme modes are enhanced with 406.7-nm (Soret) or 568.2-nm (Q band) excitation. Instrumental conditions, top to bottom (Spex 1402): power, 75, 125, 75, 100 mW; slit, 8, 7, 6, 6 cm^{-1}; scan increment, 1 cm^{-1}; integration time, 10, 40, 4, 4 s.

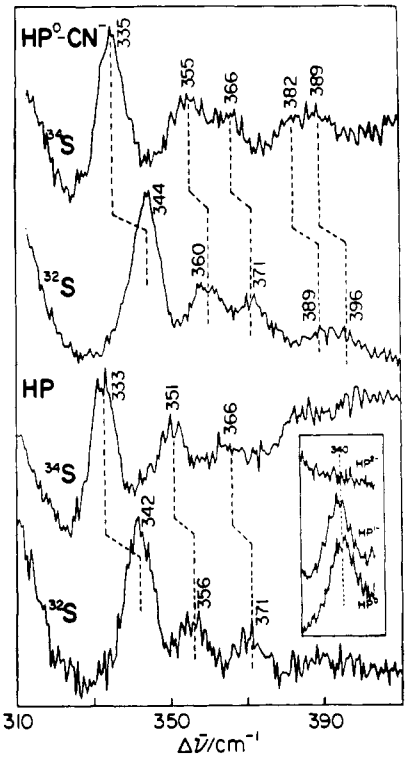

FIGURE 2: RR spectra of the iron–sulfur cluster of unlabeled and ^{34}S-labeled HP^0 (615 μM) (lower) and HP^0–CN$^-$ (512 μM) (upper) at 77 K using 457.9-nm excitation. The ferric cyanide complex was prepared by the anaerobic addition of 1.9 μL of 30 mM Cr^{II}(EDTA) reductant solution to 12 μL of 615 μM enzyme in standard buffer, followed by the addition of 0.5 μL of 250 mM potassium cyanide freshly dissolved in ice-cold 0.245 N HCl in a stoppered tube. After brief incubation under inert atmosphere, the mixture was reoxidized by exposure to air before transfer to the sample cell. Instrumental conditions (Spex 1402): power, 125 mW; slit, 7 cm^{-1}; scan increment, 1 cm^{-1}; integration time, 10 s.

Czernuszewicz et al. (1987) have recently provided rigorous assignments supported by normal coordinate calculations for the vibrational bands of Fe_4S_4 clusters in D_{2d} symmetry. The strongest Raman-active band observed, between 333 and 338 cm^{-1} in most previously described clusters, is assigned to the totally symmetric cluster bridging mode ($A_1{}^b$). The pair of modes observed around 360 cm^{-1} with 30%–60% of the intensity of the $A_1{}^b$ mode is assigned to chiefly terminal (cysteinyl–iron) vibrations ($B_2{}^t$ and E^t); these two bands collapse to a single band as the cluster symmetry approaches T_d, as appears to be the case in *Chromatium* HiPiP. A weak mode sometimes observed near 385 cm^{-1} is assigned to a bridging mode ($B_2{}^b$). Finally, a weak band near 395 cm^{-1} is assigned to a totally symmetric terminal mode ($A_1{}^t$). In the case of SiR-HP, it is apparent that the totally symmetric bridging vibration, easily identified by the band position, intensity, and large isotopic shift, is found at unusually high energy (342 cm^{-1}) compared with the clusters of ferredoxin (see Table I). The two modes in oxidized SiR-HP of approximately equal intensity at 356 and 371 cm^{-1} can then be assigned to the $B_2{}^t$ + E^t pair. These modes are also shifted to higher energy; furthermore, they display a larger splitting than in any previously observed Fe_4S_4 cluster, suggesting substantial tetragonal distortion. The highest frequency band of the SiR-HP cluster is extremely weak and occurs around 390 cm^{-1}, consistent with assignment to the $A_1{}^t$ mode. No band attributable to the $B_2{}^b$ mode is observed in the free, oxidized enzyme, but it is possibly seen at 389 cm^{-1} in the ferric cyanide derivative. In Table I these band positions are compared with those observed for a number of Fe_4S_4 proteins and analogues; the agreement of the protein ^{34}S isotopic shifts with those observed in the exhaustively ^{34}S-substituted benzyl cube analogue cluster is notable.

From the inset of Figure 2 it is apparent that the main 342-cm^{-1} cluster band (340 cm^{-1} at room temperature) is maintained when SiR-HP^0 is reduced by approximately one electron, but it disappears when a second electron is added. Reduced Fe_4S_4 (1+ state) clusters have extremely weak resonance Raman scattering; indeed, no RR spectra of clusters in this state have as yet been detected (Spiro et al., 1988). It

has been previously demonstrated by magnetic spectroscopy that in reductive titrations the siroheme is the first group in SiR-HP to be reduced, leaving the cluster oxidized, while the second titration equivalent reduces the cluster (Janick & Siegel, 1982). Thus, the RR enhancement behavior of the SiR-HP cluster during progressive reduction is entirely consistent with that of well-characterized Fe_4S_4 clusters.

The significance of the upshifts in the cluster mode frequencies is not immediately clear. In the preceding paper in this series (Han et al., 1989a) the possibility of a charge-transfer interaction from the iron–sulfur cluster to the siroheme was considered as a mechanism for explaining the low siroheme skeletal frequencies observed in SiR-HP relative to those seen in an isobacteriochlorin model. Such a charge-transfer interaction could also result in an increase in the Fe–S cluster breathing frequency due to an increase in the average Fe charge. This electronic perturbation should also be manifested in an altered Mössbauer isomer shift; this parameter is, however, quite similar in SiR-HP^0 to that found in other oxidized Fe_4S_4 clusters (Münck, 1982). The constancy of the cluster breathing frequency (see Figure 2) when the siroheme is reduced in SiR-HP^{1-} provides an additional argument against significant cluster → heme charge transfer.

Interestingly, the cluster vibrational frequencies of SiR-HP are in better agreement with those seen for Fe_3S_4 clusters than with those of four-iron ferredoxins. For example, in aconitase as isolated (Johnson et al., 1983b) the features occur at 342, 358, and 371 cm^{-1}, virtually identical with the frequencies of the SiR-HP cluster. Three-iron–four-sulfur clusters from three bacterial ferredoxins show similar frequencies (Johnson et al., 1983a,b). Since there is no doubt that SiR-HP does indeed contain an Fe_4S_4 cluster, it is tempting to speculate that the proposed connection of one Fe_4S_4 cluster iron to the siroheme through a bridging thiolate sulfur atom could, by lowering the effective cluster symmetry to approximately C_{3v}, produce a three-Fe-type vibrational signature. A similar explanation has been advanced to explain the novel anisotropy and resolution of the cluster iron magnetic hyperfine couplings in the $g = 5$ form of SiR-HP^{2-} as detected by ENDOR spectroscopy (Cline et al., 1986). Calculations based on the crystallographically determined positions of the cluster and siroheme irons clearly show that maintenance of a reasonable geometry at the putative cysteinyl bridge (vide infra) necessarily entails substantial distortion of the S^b–Fe–S^t bond angles at the involved cluster iron, from a normal value of ~114° to more than 140°. The possible effects of such distortions on cluster frequencies are not yet clearly understood. For rubredoxin, the effect of Fe–S–C–C dihedral angle on Fe–S stretching frequencies has been shown to be substantial (Yachandra et al., 1983).

Cluster Modes in Ligand Complexes of SiR-HP. Figure 3 compares the iron–sulfur cluster spectra obtained with 457.9-nm excitation for complexes of SiR-HP with several heme ligands. Small but reproducible shifts in the positions of a number of bands are observed in these adducts. In the ferric siroheme–cyanide complex (SiR-HP^0–CN^-) the resonance Raman spectrum of the cluster appears slightly perturbed, with small upshifts in two of the cluster bands at 344 and 360 cm^{-1}. In the case of the ferrous siroheme–carbonyl complex (SiR-HP^{1-}–CO) the strongest cluster band is similarly upshifted by about 2 cm^{-1} to 344 cm^{-1}, and the extremely broad and weak band around 390 cm^{-1} in the free, oxidized protein also appears downshifted by around 5 cm^{-1}. On the other hand, the ferrous siroheme–nitrosyl complex (SiR-HP^{1-}–NO) displays no alterations in the position of the three strong bands at 342, 356, and 371 cm^{-1}, although some

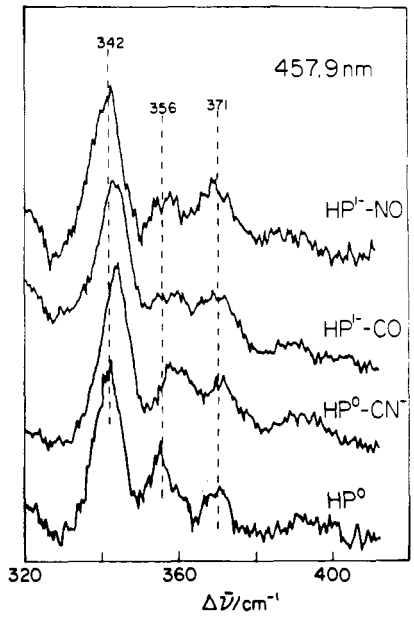

FIGURE 3: RR spectra of the iron–sulfur cluster of SiR-HP^0 and various ligand adducts at 77 K using 457.9-nm excitation. HP^0, 615 μM; HP^0–CN, 512 μM, prepared as described in legend to Figure 2; HP^{1-}–CO, 530 μM, prepared as HP^0–CN^-, except that sample was flushed with CO gas after addition of Cr^{II}(EDTA) reductant, incubated several minutes on ice, and oxidized by exposure to air before transfer to the sample cell; HP^{1-}–NO, 594 μM, prepared by addition of 11.5 μL of 29 mM Cr^{II}(EDTA) to 50 μL of 790 μM enzyme, followed by addition of 5 μL of 0.16 M $NaNO_2$ (optical λ_{max} = 398, 595). Instrumental conditions, top to bottom (Spex 1402): power, 120, 110, 125, 125 mW; slit, 7 cm^{-1}; scan increment, 1 cm^{-1}; integration time, 10, 20, 10, 20 s.

downshift of the broad, weak intensity in the 390-cm^{-1} region may be observed.

The exchange coupling between the siroheme and the cluster that is observed by magnetic spectroscopy indicates electron delocalization between the two centers, which has been proposed to be functional significance in facilitating the rapid transfer of reducing equivalents between the two sites. The Mössbauer results permit an estimate of the relative strength of the exchange coupling in complexes of SiR-HP with exogenous ligands on the basis of the magnitude of the magnetic hyperfine splitting of the cluster iron signals (Christner et al., 1983). For SiR-HP^0–CN^-, SiR-HP^0, and SiR-HP^{1-}–NO the magnitude of the exchange interaction is calculated to fall in the ratio 1:3:12, respectively. The strength of the heme–cluster coupling in the ferrosiroheme–NO complex is highest among all the characterized SiR-HP derivatives. In this connection it is of interest to note that excitation at 457.9 nm, where cluster mode enhancement is observed, also produces enhancement of the siroheme–NO vibrational mode at 558 cm^{-1}, as reported in the following paper in this series (Han et al., 1989b). The intensity of this mode relative to the siroheme ring mode intensity is higher at 457.9 nm than at 406.7 nm, in resonance with the Soret absorption band. Thus, the Fe–NO mode appears to be enhanced in resonance with an Fe_4S_4 cluster electronic transition. This phenomenon might be associated with the relatively strong heme–cluster magnetic coupling, which may reflect delocalization of the Fe_4S_4 excited state, and a nonnegligible origin shift along the Fe–NO coordinate. On the other hand, the Raman results demonstrate no consistent correlation between the magnitude of the exchange coupling and the frequencies of the cluster modes. For the ferric cyanide derivative, the positions of both the A_1 bridging mode and the T_2 terminal mode of the cluster are shifted to slightly higher frequency than in the free, oxidized

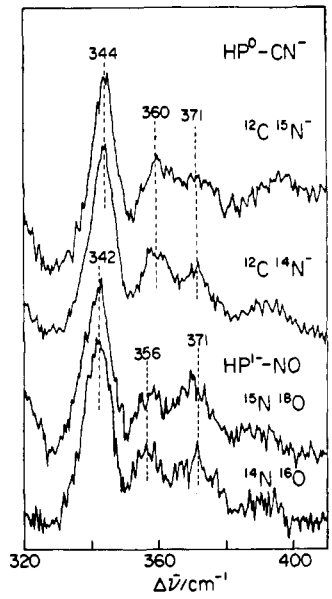

FIGURE 4: RR spectra of isotopomers of SiR-HP^{1-}–NO and SiR-HP^{0}–CN^{-} at 77 K obtained with 457.9-nm excitation. Samples were prepared and spectra obtained as described in legend to Figure 3.

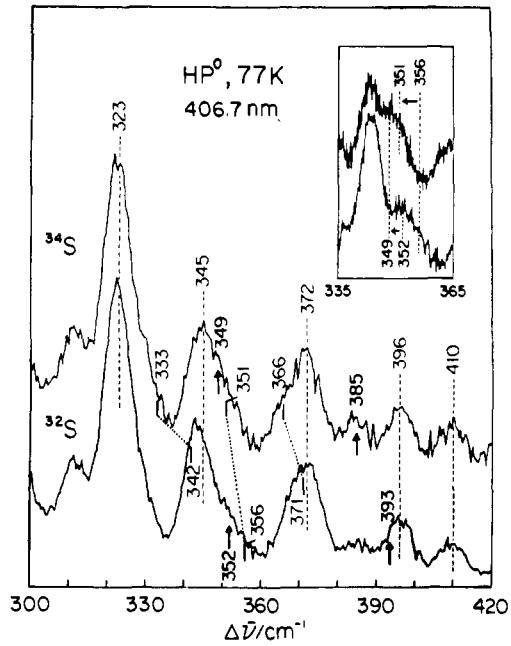

FIGURE 5: RR spectra of unlabeled (see legend to Figure 1) and ^{34}S-labeled HP^{0} (615 μM) in standard buffer at 77 K using 406.7-nm excitation. Instrumental conditions, ^{34}S-labeled sample (Spex 1402): power, 75 mW; slit, 6 cm^{-1}; scan increment, 1 cm^{-1}; integration time, 8 s. (Inset) High-resolution scan of the 335–365-cm^{-1} region using 413.1-nm excitation. Instrumental conditions (Spex 1402): power, 180 mW; slit, 3.5 cm^{-1}; scan increment, 0.1 cm^{-1}; integration time, 8 s.

enzyme, indicating a measurable effect on the cluster modes of ligand binding to the heme. However, no such difference is noted when the free, oxidized enzyme is compared to the ferrous nitrosyl complex; in these two complexes the observable cluster vibrational frequencies are identical. Furthermore, the relatively small magnitude of the shifts would make it appear that dramatic structural changes in the ligand environment of the cluster are not necessary to accommodate alterations in the electronic interaction between the cluster and the heme that span more than an order of magnitude. Christner et al. (1983) have speculated that the differences in the magnitude of the coupling between the uncomplexed enzyme and the NO derivative might represent actual replacement of the endogenous bridging ligand by NO itself. The absence of any perturbation of the cluster Fe–S terminal mode vibrational frequencies at 356 and 371 cm^{-1} in this derivative is evidence against this hypothesis.

Further evidence against direct binding of exogenous ligands to the cluster is presented in Figure 4, in which the cluster spectra of the normal ferric cyanide and ferrous nitrosyl derivatives are compared with those of the same complexes formed by using isotopically substituted ligands. No differences are detected in the band positions of the cluster modes in the $^{12}C^{14}N^{-}$ complex compared with the $^{12}C^{15}N^{-}$ complex, nor in the $^{14}N^{16}O$ complex compared with the $^{15}N^{18}O$ complex. Shifts in these bands would have been anticipated if these ligands were bound directly to the cluster.

Axial Linkage. The experiment illustrated in Figure 4 was one of many that failed to provide clear-cut evidence for a siroheme Fe–S stretching mode. Identification of such a mode was naturally a prime target of our research since it would provide direct evidence in support of the proposal that the cluster and siroheme are bridged by a common sulfur ligand (Christner et al., 1984). Such a mode might be expected to be detectable by virtue of enhancement with excitation into siroheme absorption bands in addition to or in preference to cluster absorption bands. Initially we therefore hypothesized that the strong 323-cm^{-1} band seen in the 406.7-nm-excited RR spectrum (see Figure 1) might represent such a mode. This frequency is not unreasonable considering that the heme Fe^{III}–thiolate stretch has been identified at 351 cm^{-1} in the RR spectrum of cytochrome P450 (Champion et al., 1982) and at 347 cm^{-1} of chloroperoxidase (Bangcharoenpaurpong et al., 1986). A somewhat lower frequency might be expected for tricoordinate sulfur, consistent with the location of ν(Fe–SR_2) at 328 cm^{-1} in the IR spectrum of Fe^{III}(TPP)(THT)$_2$ (THT = tetrahydrothiophene) (Oshio et al., 1985). However, the 323-cm^{-1} band failed to show any frequency shift in the ^{34}S-labeled protein, although the proper shifts were observed for the Fe_4S_4 cluster, as discussed above. RR spectra obtained with several other excitation wavelengths likewise failed to reveal any prominent ^{34}S-sensitive band, other than the cluster bands. In particular, near-UV excitation wavelengths between 330 and 364 nm, where selective enhancement for the cytochrome P450 Fe–thiolate stretch has been reported (Champion et al., 1982), were attempted with negative results. These wavelengths are thought to be in resonance with a thiolate–Fe^{III} charge-transfer transition in cytochrome P450 and chloroperoxidase. In SiR-HP^{0}, the proposed bridging sulfur atom is bound to a cluster Fe as well as the siroheme Fe. As a result, significant electronic perturbation is anticipated, and it was not unreasonable that enhancement at these wavelengths should have been ineffective.

Careful examination of the ^{32}S and ^{34}S protein spectra with violet excitation (406.7 or 413.1 nm) does, however, suggest a candidate for the axial Fe–S stretch. These spectra, shown in Figure 5, are dominated by siroheme modes, but the Fe_4S_4 cluster modes, which have been separately cataloged in the RR spectra obtained with blue excitation as detailed above, can be seen as shoulders via their ^{34}S shifts. A high-resolution scan of the 350-cm^{-1} region (see Figure 5, inset) indicates, however, that the 356-cm^{-1} cluster mode does not fully account for the ^{34}S-sensitive shoulder on the 345-cm^{-1} siroheme band, since the main intensity of the shoulder is at a detectable lower frequency in both the ^{32}S and ^{34}S spectra. As indicated in the inset, the spectral shape is best accounted for if there is an additional band at 352 cm^{-1}, which shifts to 349 cm^{-1} in the ^{34}S protein. This could be a siroheme Fe–S stretching mode.

The appearance of this feature in the spectra obtained with violet but not with blue excitation would be a logical consequence of greater coupling (albeit still weak) of the siroheme Fe–S stretch with the siroheme π–π^* transition (Soret band) than with the iron–sulfur cluster charge-transfer transitions.

We note in addition that the intensity of the 385-cm^{-1} band in the ^{34}S spectrum, which is assigned to a downshifted cluster mode, is greater than expected on the basis of the cluster spectra obtained with blue excitation (see Figure 2). The ^{32}S protein has a broad ~390-cm^{-1} band that is expected to have both bridging and terminal contributions (Table I). This band appears to be resolved into 389- and 396-cm^{-1} components in HP^0–CN^- (Figure 2); both components shift down ~7 cm^{-1} in the ^{34}S protein. These are the weakest bands in the cluster spectrum and are barely resolved from the background in HP^0 (Figure 1). With 406.7-nm excitation (Figure 5) the ~390-cm^{-1} cluster bands are obscured in the ^{32}S protein by a porphyrin band at 396 cm^{-1}. But the ^{34}S downshift reveals at least one component of the cluster band, at 385 cm^{-1}. This band is nearly as strong as the other cluster bands in the 406.7-nm spectrum, suggesting that it too is differentially enhanced via the siroheme Soret transition.

It seems possible that this 385-cm^{-1} band is a component of the terminal Fe–S cluster mode which is coupled to the siroheme Fe–S stretch via the bridging sulfur atom. Indeed, the two modes in question could be considered as antisymmetric and symmetric stretches, respectively, of the Fe–S bonds connecting the bridging thiolate S atom with the siroheme Fe on the one hand and a cluster Fe on the other. We have modeled this pair of modes with an Fe–S–Fe triatomic oscillator and have calculated the expected ^{34}S isotope shifts as a function of the bridge angle. The observed isotope shifts for the 352- and 393-cm^{-1} features are 3 and 8 cm^{-1}, respectively. The best agreement with these values is obtained for a Fe–S–Fe bond angle of 140° (the calculated shifts are 3 and 9 cm^{-1}, respectively, with stretch and bend force constants K_{Fe-S} = 2.07 and F = 0.83 mdyn/Å). Given the 4.4-Å separation between the siroheme iron and the nearest cluster iron reported in the crystallographic study (McRee et al., 1986), an approximate calculation of the $Fe_{cluster}$–S(R)–$Fe_{siroheme}$ bridging bond angle is possible. On the basis of a siroheme Fe–S distance of 2.44 Å [in analogy to the known distance in the crystallographically characterized five-coordinate heme Fe^{III}–S(R)–Cu^{II} complex (Schauer et al., 1984)] and assuming a typical value of 2.26 Å for the cluster iron–terminal sulfur bond (Stout, 1982), a value of 139° for this bridge angle is obtained. The agreement with the value inferred from the model calculation is excellent.

Conclusions. Excitation of sulfite reductase hemoprotein with blue laser lines (457.9 and 488.0 nm) selectively enhances the Fe_4S_4 cluster RR vibrational modes of SiR-HP, as confirmed by the ^{34}S isotope shifts of the cluster modes. The breathing mode frequency is slightly but significantly higher than in other Fe_4S_4 clusters so far observed. The discrepancy is unlikely to be due to the electronic interaction of the cluster with the siroheme, since the frequency is unaffected by reduction to Fe^{II} siroheme. It may result from a structural alteration of the cluster itself involving the bond angles of the terminal ligands; the presence of unusual geometric constraints at one terminal cluster ligand is a likely consequence of the proposed cysteinyl siroheme–cluster bridge. Interaction of the cluster with the siroheme is demonstrated by a slight but detectable upshift in the breathing mode and a terminal mode frequency when CN^- is bound to the siroheme. However, the small magnitude of these shifts in a number of ligand complexes of the enzyme and the insensitivity of the cluster spectra to isotopically substituted NO and CN^- ligands argue against a model of ligand binding involving replacement of an endogenous heme–cluster bridge by substrate. The symmetric and antisymmetric stretching modes of the bonds connecting the putative bridging thiolate ligand with the siroheme Fe and the cluster Fe may lie at 352 and 393 cm^{-1}; while consistent with model calculations, the weakness of the ^{34}S-sensitive spectral features observed at these frequencies makes this a tentative proposal.

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Resonance Raman Studies of *Escherichia coli* Sulfite Reductase Hemoprotein. 3. Bound Ligand Vibrational Modes†

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ABSTRACT: The vibrations of the bound diatomic heme ligands CO, CN^-, and NO are investigated by resonance Raman spectroscopy in various redox states of *Escherichia coli* sulfite reductase hemoprotein, and assignments are generated by use of isotopically labeled ligands. For the fully reduced CO complex (ferrous siroheme, reduced Fe_4S_4 cluster) at room temperature, νCO is observed at 1904 cm^{-1}, shifting to 1920 cm^{-1} upon oxidation of the cluster. The corresponding δFeCO modes are identified at 574 and 566 cm^{-1}, respectively, by virtue of the zigzag pattern of their isotopic shifts. In frozen solution, two species are observed for the cluster-oxidized state, with νCO at 1910 and 1936 cm^{-1} and νFeC at 532 and 504 cm^{-1}, respectively; νFeC for the fully reduced species is identified at 526 cm^{-1} in the frozen state. For the ferrous siroheme–NO complex (cluster oxidized), νNO is identified at 1555 cm^{-1} in frozen solution and a low-frequency mode is identified at 558 cm^{-1}; this stretching mode is significantly lower than that observed in Mb–NO. For the ferric siroheme cyanide complexes evidence of two ligand-bonding forms is observed, with modes at 451/390 and 451/352 cm^{-1}; they are distinguished by a reversal of the isotopic shift patterns of the upper and lower modes and could arise from a linear and a bent Fe–C–N unit, respectively. For the ferrous siroheme cyanide complex isotope-sensitive modes observed at 495 and 452 cm^{-1} are assigned to the $FeCN^-$ bending and FeC stretching vibrations, respectively. The possible origin of unusual features of these bound ligand spectra is considered. Distal H-bonding may contribute to these effects, on the basis of the similarity of the reduced CO species to those of an H-bonded form of the CO adduct of horseradish peroxidase.

The hemoprotein subunit (SiR-HP) from *Escherichia coli* sulfite reductase contains a siroheme and an Fe_4S_4 cluster in proximity, as established by X-ray crystallography (McCree et al., 1986). They are magnetically coupled (Janick & Siegel, 1983), and it has been suggested that they are bridged by a common thiolate ligand. In previous papers we have examined the resonance Raman scattering from the siroheme (Han et al., 1989) and the Fe_4S_4 cluster (Madden et al., 1989) in the free enzyme and in several of its stable adducts with exogenous ligands.

On the distal side of the siroheme, opposite the Fe_4S_4 cluster, the X-ray crystal structure does not show significant electron density (McRee et al., 1986). This may be the site where ligands bind to the siroheme, including the substrates SO_3^{2-} and NO_2^-. In the present study we examine RR bands arising from vibrational modes of X–Y diatomic ligands (CO, NO, and CN^-) bound to the siroheme. These include the Fe–X and X–Y stretching modes as well as the Fe–X–Y bending mode. These modes have previously been detected in heme proteins and in protein-free heme complexes, especially in the pioneering studies of Yu and co-workers (Yu, 1986; Kerr & Yu, 1988), via their characteristic isotope shifts. Their enhancement in resonance with the heme electronic transitions is attributed to coupling with the π–π^* excited states. The frequencies and intensities of these modes have been found to provide useful probes of the interactions of the distal ligands with protein side chains around the distal pocket. In particular, distal H-bonding to bound CO produces a downshift in the CO stretching frequency but an upshift in the Fe–C stretching frequency due to the enhanced back-bonding induced by the H-bond interaction (Smulevich et al., 1986; Uno et al., 1987).

In sulfite reductase we find clear evidence for strong perturbation of bound diatomic ligands, probably via distal interactions that may involve H-bonding. An important role for such an interaction is anticipated on the basis of a consideration of the mechanistic requirements of sulfite reduction.

Experimental Procedures

Reagents. $K^{13}CN^-$ (90%), $KC^{15}N$ (99%), $Na^{15}NO_2$ (99%), and ^{13}CO (99%) were from Stohler Isotope Chemicals (Cambridge, MA). $K^{13}C^{15}N$ (99%/99%), $C^{18}O$ (99%), and $^{13}C^{18}O$ (99%/99%) were from Icon Services (Summit, NJ).

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