Hydrogen Bonding of Sulfur Ligands in Blue Copper and Iron–Sulfur Proteins: Detection by Resonance Raman Spectroscopy†

Yoshiki Mino,‡,§ Thomas M. Loehr,‡ Keishiro Wada,∥ Hiroshi Matsubara,∥ and Joann Sanders-Loehr*,‡

Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999, and Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan

Received May 14, 1987; Revised Manuscript Received July 30, 1987

ABSTRACT: The resonance Raman spectrum of the blue copper protein azurin from *Alcaligenes denitrificans* exhibits nine vibrational modes between 330 and 460 cm^{-1}, seven of which shift 0.4–3.0 cm^{-1} to lower energy after incubation of the protein in D_2O. These deuterium-dependent shifts have been previously ascribed to exchangeable protons on imidazole ligands [Nestor, L., Larrabee, J. A., Woolery, G., Reinhammar, B., & Spiro, T. G. (1984) *Biochemistry 23*, 1084] or to exchangeable protons on amide groups which are hydrogen bonded to the cysteine thiolate ligands (a feature common to all blue copper proteins of known structure). In order to distinguish between these two possibilities, a systematic investigation of $Fe_2S_2(Cys)_4$-containing proteins was undertaken. Extensive hydrogen bonding between sulfur ligands and the polypeptide backbone had been observed in the crystal structure of ferredoxin from *Spirulina platensis*. The resonance Raman spectrum of this protein is typical of a chloroplast-type ferredoxin and exhibits deuterium-dependent shifts of −0.3 to −0.5 cm^{-1} in the Fe-S modes at 283, 367, and 394 cm^{-1} (assigned to the bridging sulfurs) and −0.6 to −0.8 cm^{-1} in the Fe-S modes at 328 and 341 cm^{-1} (assigned to the terminal cysteine thiolates). Considerably greater deuterium sensitivity is observed in the Raman spectra of spinach ferredoxin and bovine adrenodoxin, particularly for the symmetric stretching vibration of the Fe_2S_2 moiety at ~390 cm^{-1}. This feature decreases by 0.8 and 1.1 cm^{-1}, respectively, for the two oxidized proteins in D_2O and by 1.8 cm^{-1} for reduced adrenodoxin in D_2O. These results suggest that the bridging sulfido groups may be more extensively hydrogen bonded in spinach ferredoxin and adrenodoxin than in *S. platensis* ferredoxin, with a further increase in hydrogen-bond strength in the reduced form of adrenodoxin. The similarity of the deuterium effects in the iron–sulfur and blue copper proteins indicates that the deuterium dependence of the blue copper Raman spectra in the 330–460-cm^{-1} region is mainly due to hydrogen-bonded cysteinate ligands.

Blue copper proteins are redox-active proteins that cycle between Cu(I) and Cu(II) and that exhibit an unusually intense visible absorption band, $\epsilon_{620} \simeq 4000$ M^{-1} cm^{-1}, in the Cu(II) state (Solomon et al., 1983). The structures of the Cu(II) centers have been determined by X-ray crystallography for poplar plastocyanin (Guss & Freeman, 1983) and for the azurins from *Pseudomonas aeruginosa* (Adman, 1985) and *Alcaligenes denitrificans* (Norris et al., 1986). In each case the Cu(II) is found in a distorted tetrahedral- or trigonal-bipyramidal environment, strongly coordinated to one cysteine thiolate and two histidine nitrogens. Alcohol dehydrogenase, which also contains a tetrahedrally organized set of ligands (two Cys, one His, one H_2O) in the native zinc form (Eklund & Brändén, 1983), exhibits spectroscopic properties similar to those of the blue copper protein when its Zn(II) is replaced by Cu(II) (Maret et al., 1986). For both plastocyanin and alcohol dehydrogenase, the pseudotetrahedral arrangement of ligands at the metal binding site is essentially unaffected by removal of the metal ion or by replacement with other divalent metals (Garrett et al., 1984; Church et al., 1986; Schneider et al., 1983, 1985). These results suggest that the metal ligands are held in a fairly rigid framework by the protein and that this imposed coordination geometry is important to the biological activity of the metal center.

The intense optical absorption of the blue copper proteins has been ascribed to a cysteinate sulfur → Cu(II) charge-transfer (CT) transition (Solomon et al., 1983). Excitation within this absorption band produces a resonance Raman spectrum containing four or more strongly enhanced vibrational modes in the 350–450-cm^{-1} region (Woodruff et al., 1987). The smaller-than-expected copper isotope dependence of these peaks has led to the conclusion that they represent coupling between Cu–L stretches and internal ligand vibrations (Nestor et al., 1984; Blair et al., 1985). It is likely that the Cu–S stretch of cysteine makes a contribution to each of these normal modes because all of the Raman peaks show enhancement profiles that faithfully track the ~620-nm S(Cys) → Cu(II) CT transition (Ainscough et al., 1987; Musci et al., 1985). The unusually short Cu–S bonds (<2.20 Å) in the blue copper proteins also help to explain the unusually high energies of these Cu–S + cysteinate vibrations. Since many of the spectral peaks in the 350–450-cm^{-1} region show distinctive shifts of −1 to −2 cm^{-1} upon deuteriation of the protein, additional coupling to imidazole ligand modes has been proposed (Nestor et al., 1984). However, considerably more anomalous deuterium isotope effects including changes in intensity and increases in frequency have been observed in the resonance Raman spectrum of Cu(II)-substituted alcohol dehydrogenase in its coenzyme-bound form (Maret et al., 1986). This behavior is characteristic of hydrogen-bonded systems where the vibrational frequency of the hydrogen-bond acceptor can either increase or decrease depending on whether the hydrogen bond is weaker or stronger with deuterium (Buckingham & Fan-Chen, 1981). These findings led to the hypothesis that the deuterium isotope effects in blue copper proteins are due to

† This research was supported by the National Institutes of Health, U.S. Public Health Service, Grant GM-18865 (T.M.L. and J.S.-L.).

* Author to whom correspondence should be addressed.

‡ Oregon Graduate Center.

§ Present address: Osaka University of Pharmaceutical Sciences, Matsubara-City, Osaka 580, Japan.

∥ Osaka University.

0006-2960/87/0426-8059$01.50/0

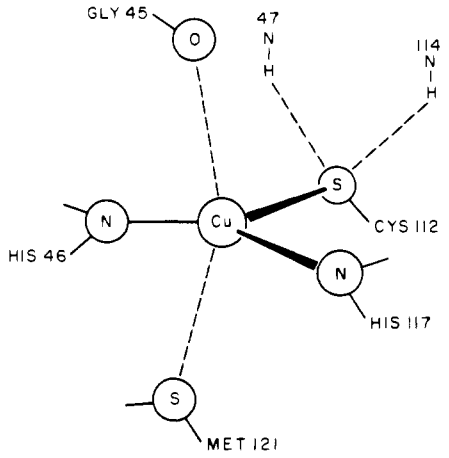

FIGURE 1: Blue copper center of azurin from *A. denitrificans*. Dotted lines depict weakly coordinated copper ligands and hydrogen bonding of the cysteine sulfur ligand to backbone amides (Ainscough et al., 1987; E. N. Baker, personal communication).

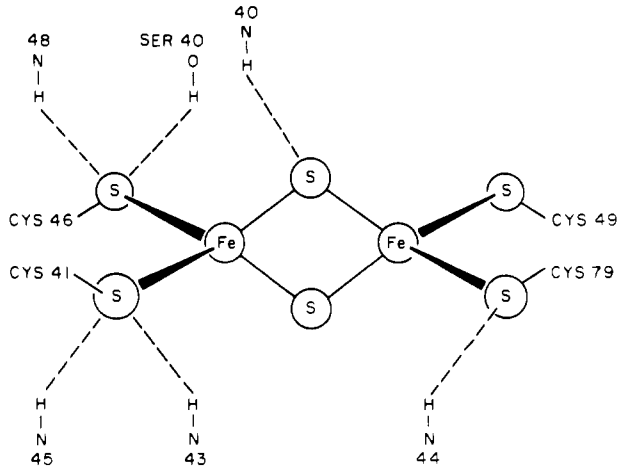

FIGURE 2: Binuclear iron–sulfur cluster of ferredoxin from *Spirulina platensis*. Dotted lines depict hydrogen bonding of bridging and terminal sulfur ligands to backbone amides and serine OH [redrawn from Tsukihara et al. (1986)].

hydrogen bonding of the cysteinate sulfur atom to the protein backbone (Maret et al., 1986; Ainscough et al., 1987).

Hydrogen bonding of internal polar groups either to other protein moieties or to immobilized water molecules is a ubiquitous characteristic of protein structures (Baker & Hubbard, 1984). The polar character of the sulfur ligands in metalloproteins makes them likely, albeit weak, hydrogen-bond acceptors (Pogorely, 1977). In plastocyanin the sulfur of the Cys-84 ligand is hydrogen bonded to the amide NH of residue 38 (Guss & Freeman, 1983), while in azurin from *A. denitrificans* the sulfur of the Cys-112 ligand is actually involved in two hydrogen bonds to the protein backbone (Figure 1). In the native zinc form of liver alcohol dehydrogenase, the sulfur of the Cys-46 ligand is also hydrogen bonded to a main-chain NH (48) (H. Eklund, personal communication). Such hydrogen bonds may be important in helping to orient the ligands for specific coordination geometries.

Hydrogen bonds between thiolate sulfur ligands and polypeptide NH groups have also been regularly observed in X-ray crystal structures of iron–sulfur proteins. In the $Fe(Cys)_4$ center of *Clostridium pasteurianum* rubredoxin, for example, there are six hydrogen bonds between the NH groups of amides in the polypeptide chain and the sulfurs of the cysteine ligands (Adman et al., 1975). Similarly, the $Fe_2S_2(Cys)_4$ cluster of *Spirulina platensis* ferredoxin exhibits five hydrogen bonds between the cysteine sulfurs and the protein and one hydrogen bond between a bridging sulfide and the protein (Figure 2). In *Peptococcus aerogenes* ferredoxin, each of the $Fe_4S_4(Cys)_4$ clusters exhibits nine hydrogen bonds (Adman et al., 1975), while in the $Fe_4S_4(Cys)_4$ cluster of *Chromatium vinosum* high-potential iron–sulfur protein the ligating sulfur atoms are involved in a total of six hydrogen bonds (Carter et al., 1974; Sheridan et al., 1981). Since there are no histidine ligands associated with any of these iron–sulfur centers, these proteins offer the possibility of investigating the sole effects of hydrogen bonding to sulfur ligands in the resonance Raman spectrum. In the present study of three Fe_2S_2-containing proteins, spinach ferredoxin, *S. platensis* ferredoxin, and adrenodoxin, we have been able to observe deuterium-dependent Raman shifts similar to those found in the blue copper proteins. Thus, it would appear that hydrogen bonding alone can account for such occurrences.

Experimental Procedures

Spinach Ferredoxin. The protein was isolated and purified according to the procedure of Dr. John H. Golbeck of Portland State University. An aqueous extract of ground spinach was centrifuged to remove membranous material. The supernatant was adsorbed onto 10 g of DEAE-cellulose (DE-52, Whatman) by stirring for 2 h and then eluted with 500 mL of 0.02 M tris(hydroxymethyl)aminomethane (Tris) containing 1 M KCl (pH 8.3). The eluant was concentrated by ultrafiltration (YM-05 membrane, Amicon) and applied to a column (1.5 × 90 cm) containing Sephadex G-75 (10–40 μm) in 0.02 M Tris containing 0.1 M KCl (pH 7.6). Peak fractions based on A_{420} were pooled, diluted with 500 mL of 0.02 M Tris-HCl (pH 7.6), and applied to a DE-52 column (1.5 × 30 cm). The ferredoxin was eluted with a 500-mL linear gradient of 0–0.8 M KCl in 0.02 M Tris-HCl (pH 7.6). Peak fractions were again concentrated by ultrafiltration and chromatographed on Sephadex G-75, yielding a final purity ratio (A_{420}/A_{276}) of 0.37. Samples for Raman spectroscopy in 0.02 M Tris-HCl (pH 7.6) were concentrated in a Centricon 10 ultrafiltration unit (Amicon). The Centricon was also used for deuterium isotope exchange by two cycles of 10-fold dilution in 0.02 M Tris-HCl in D_2O (pH meter reading 7.8), followed by reconcentration. The protein was equilibrated in this buffer for at least 4 days at 5 °C to ensure complete exchange of protons close to the iron–sulfur site (Orme-Johnson et al., 1983). The H_2O control sample was similarly treated.

Azurin. The protein was isolated from *A. denitrificans* NCTC 8582 as described previously (Ainscough et al., 1987). Deuterium isotope exchange and sample concentration were accomplished by ultrafiltration.

Adrenodoxin. The protein was purified from bovine adrenal glands (Pel-Freeze) according to the method of Orme-Johnson and Beinert (1969). Minor modifications were that ultrafiltration was used instead of a small DEAE-cellulose column for the concentration of the protein and that instead of electrophoresis the final purification was carried out by a combination of DE-52 column chromatography using a 500-mL linear gradient elution of 0.1–0.5 M KCl in 0.05 M Tris-HCl (pH 8.1) and gel filtration using Sephadex G-75 in 0.05 M Tris-HCl (pH 8.1). The final purity ratio (A_{415}/A_{280}) was 0.76. Deuterium exchange was performed on the oxidized protein in 0.05 M Tris-HCl (pH meter reading 8.3) as described for spinach ferredoxin. Reduction was accomplished with anaerobic protein (0.5–1.0 mM) in H_2O or D_2O by the addition of a 2-fold molar excess of $Na_2S_2O_4$ (1 mM).

Spirulina Ferredoxin. Ferredoxin from *S. platensis* was purified by ammonium sulfate fractionation and DEAE-cel-

Table I: Deuterium Isotope Shifts in Raman Spectra of Iron–Sulfur Proteins[a]

ferredoxin (spinach)		ferredoxin (*Spirulina*)		adrenodoxin (oxidized)		adrenodoxin (reduced)		
ν	$\Delta\nu$	ν	$\Delta\nu$	ν	$\Delta\nu$	ν	$\Delta\nu$	calcd ν and assignment[b]
283.8	−0.3	283.2	−0.2	289.2	−0.3	275	0	286, B_{1u} (b)
				315.9	0			310, A_{1g} (t)
329.0	−0.4	328.3	−0.5	328.4	−1.1			338, B_{3g} (t)
				340.2	−1.4			338, B_{2u} (t)
337.7	−2.0	340.8	−0.7	349.9	−0.9			360, B_{1u} (t)
366.0	−1.0	367.0	−0.6					345, B_{2g} (b)
392.8	−0.8	393.7	−0.2	391.3	−1.1	375.8	−1.8	398, A_{1g} (b)
424.6		425.0		419.4		397		411, B_{3u} (b)

[a] ν in cm^{-1}. $\Delta\nu$ for frequency in D_2O minus frequency in H_2O. Spectral conditions as in Figures 4–6. Peak positions in H_2O and D_2O determined by curve fitting. [b] Based on D_{2h} symmetry (Yachandra et al., 1983); (b) = bridging Fe–S_b modes, and (t) = terminal Fe–S_t(Cys) modes.

lulose chromatography as reported previously (Wada et al., 1974), with the exception that the protein was concentrated by ultrafiltration. The final purity ratio (A_{422}/A_{275}) was 0.35 in 0.05 M Tris-HCl (pH 8.0). Deuterium exchange was achieved by incubating the oxidized protein in 0.05 M Tris-HCl in D_2O (pH meter reading 8.2) for 4 or 14 days, followed by ultrafiltration as described for spinach ferredoxin.

Resonance Raman Spectroscopy. Raman spectra were collected on a computer-interfaced Jarrell-Ash spectrophotometer (Loehr et al., 1979) equipped with Spectra-Physics 164-05 (Ar) and 2025-11 (Kr) lasers, an RCA C31034A photomultiplier tube, and an ORTEC Model 9302 amplifier/discriminator. Samples were maintained at 15 K in a closed-cycle helium Displex (Air Products) or at 90 K with a copper cold-finder in a Dewar cooled with liquid N_2 (Sjöberg et al., 1982). Data were collected in a 150° backscattering geometry. Isotope comparisons were performed on spectra collected under identical conditions. In each case peak positions were determined by abscissa expansion and curve fitting (typical values: 70% Guassian, 30% Lorentzian, full width at half-height of 7–11 cm^{-1}). Although absolute peak frequencies are accurate to only ±1 cm^{-1}, isotope shifts ($\Delta\nu$) for well-resolved features are reproducible to within ±0.2 cm^{-1}.

Computer Graphics. Atomic coordinates for ferredoxin from *S. platensis* (Tsukihara et al., 1981) were taken from the Brookhaven Protein Data Bank. The capability of protein residues to hydrogen bond to sulfur atoms in the iron–sulfur center was estimated with an Evans and Sutherland interactive computer graphics system (courtesy of Dr. Ronald E. Stenkamp, University of Washington). Donor-sulfur orientations exhibiting N–H···S or O–H···S angles ≥ 100° and N(H)···S or O(H)···S distances ≤ 4.0 Å were considered to be likely candidates for hydrogen-bond formation.

Results

The resonance Raman spectrum of azurin from *A. denitrificans* is shown in Figure 3. It exhibits nine well-resolved components between 320 and 480 cm^{-1}. As discussed above, the intense features are believed to arise from vibronic coupling between the Cu–S and internal vibrations of the single cysteine ligand. Seven of these peaks undergo distinctive shifts of −0.4 to −3.0 cm^{-1} upon deuterium exchange. This pattern of deuterium isotope sensitivity is typical of the Raman spectra of blue copper proteins (Nestor et al., 1984; Blair et al., 1985).

The resonance Raman spectrum of the $Fe_2S_2(Cys)_4$ center of oxidized spinach ferredoxin (Figure 4) is similar to those reported previously (Yachandra et al., 1983; Meyer et al., 1986). This spectrum has been assigned to a set of coupled Fe–S stretching vibrations involving either the bridging sulfides or the terminal cysteine thiolates (Table I) by (i) comparison with Fe_2S_2-containing compounds, (ii) quantitation of isotope shifts accompanying sulfide bridge substitution, (iii) excitation profiles, and (iv) normal coordinate analysis (Yachandra et

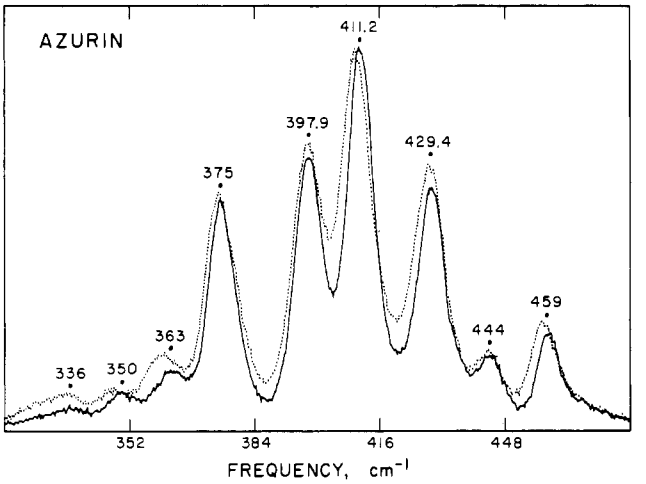

FIGURE 3: Resonance Raman spectra of *A. denitrificans* azurin in H_2O (—) and D_2O (···). Samples were 1 mM protein in 0.05 M phosphate (pH 6.5 in H_2O, pH reading 6.1 in D_2O). Spectra were obtained at 90 K with 647.1-nm excitation (160 mW), 4-cm^{-1} resolution, and 0.5 cm^{-1}/s scan rate and represent the accumulation of 25 scans without smoothing. Indicated peak frequencies are for the protein in H_2O. Shifts in D_2O in cm^{-1} are −1 (336), −2 (350), −3 (363), −0.4 (375), −1.0 (411), −0.4 (429), and −1.4 (459) (Ainscough et al., 1987).

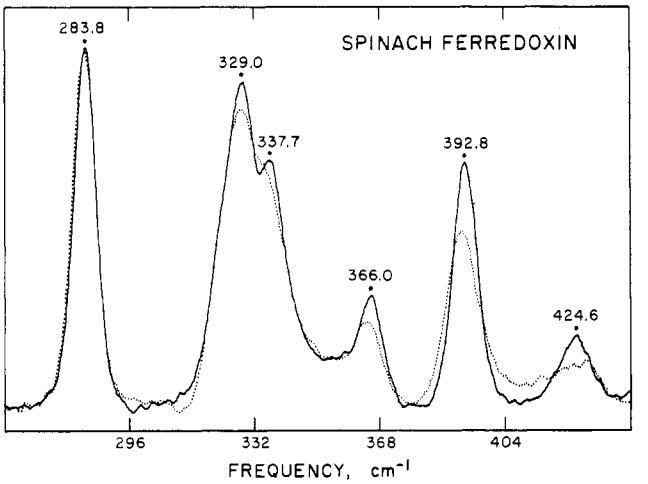

FIGURE 4: Resonance Raman spectra of oxidized spinach ferredoxin in H_2O (—) and D_2O (···). Samples were 2–3 mM in 0.02 M Tris-HCl (pH 7.6 in H_2O, pH reading 7.8 in D_2O). Spectra were obtained at 15 K with 488.0-nm excitation (250 mW at the sample) with 5-cm^{-1} spectral resolution, 1.0 cm^{-1}/s scan rate, 16 scans, and a 13-point smooth. Contributions from frozen solvent in the 220–310-cm^{-1} region (Maret et al., 1986) have been removed by subtracting out the spectrum of the sample buffer. Indicated peak frequencies are for the protein in H_2O.

al., 1983; Beardwood & Gibson, 1984). The peaks at 283.8, 366.0, 392.8, and 424.6 cm^{-1} appear to be associated primarily with Fe–S_b vibrations of the bridging sulfides in the Fe_2S_2 core, while those at 329.0 and 337.7 cm^{-1} are ascribed to Fe–S_t vibrations of the terminal thiolates.

The spinach ferredoxin spectrum displays significant changes after prolonged incubation of the protein in D_2O (Figure 4). The Fe–S_b peaks show shifts of −0.3 cm^{-1} at 283.8 cm^{-1} and ca. −1.0 cm^{-1} at 366.0 and 392.8 cm^{-1}, while the Fe–S_t peaks have undergone shifts of −0.4 cm^{-1} at 329.0 cm^{-1} and −2.0 cm^{-1} at 337.7 cm^{-1} (Table I). In addition, the peaks at 329.0, 337.7, 366.0, and 392.8 cm^{-1} have decreased intensities relative to the peak at 284 cm^{-1} in the deuterium-treated protein. The Fe–S_b peak at 424.6 cm^{-1} appears to have a higher as well as a lower energy component, but the lowered intensity of this feature makes it difficult to resolve. Although the state of the iron–sulfur cluster in spinach ferredoxin as measured by redox potential has previously been shown to be sensitive to pH (Magliozzi et al., 1982), a comparison of Raman spectra taken between pH 7.0 and pH 8.0 in H_2O revealed no significant changes. Thus, the deuterium isotope effect in spinach ferredoxin is more likely due to hydrogen bonding of the iron–sulfur cluster than to possible protein ionization differences in H_2O and D_2O.

In order to determine whether deuterium dependence is a general feature in Raman spectra of binuclear iron–sulfur proteins, similar experiments were performed with adrenodoxin and a cyanobacterial ferredoxin. The spectrum of oxidized adrenodoxin presented in Figure 5 resembles examples in the literature (Yachandra et al., 1983). The general pattern of the Raman spectrum is similar to that of spinach ferredoxin, but there are substantial differences in peak positions and relative intensities, particularly involving the Fe–S_t modes at 315.9, 328.4, 340.2, and 349.9 cm^{-1} in adrenodoxin and the disappearance of the 366.0-cm^{-1} Fe–S_b mode of spinach ferredoxin. The effects of deuterium exchange on oxidized adrenodoxin are remarkably close to those in spinach ferredoxin. Of the Fe–S_b peaks, there is a small −0.3-cm^{-1} shift at 289.2 cm^{-1} and a −1.1-cm^{-1} shift at 391.3 cm^{-1}, while the Fe–S_t peaks show shifts of −0.9 to −1.4 cm^{-1} at 328.4, 340.2, and 349.9 cm^{-1} (Table I). The 419.4-cm^{-1} peak again shows substantial broadening upon deuterium substitution, suggestive of both higher and lower energy components. Although the peaks between 328.4 and 391.3 cm^{-1} have decreased heights relative to the peak at 289.2 cm^{-1}, the corresponding increases in peak widths lead to a less significant decrease in peak areas than was the case with spinach ferredoxin. The shoulder at 315.9 cm^{-1} actually appears to have gained intensity in D_2O. The observed deuterium isotope effects support the existence of a hydrogen-bonding network in adrenodoxin and suggest that it is similar to that of spinach ferredoxin.

The one-electron reduction of adrenodoxin to the trapped valence Fe(III) + Fe(II) state (Thompson, 1985) is accompanied by a diminution in the intensity of the sulfur → Fe(III) CT bands, with the strongest remaining feature now at longer wavelength (~550 nm). As a result, Raman peaks are less resonance enhanced, and the overall spectral quality is poorer. Nevertheless, three distinct features can still be observed at 275, 375.8, and 397 cm^{-1} (Figure 5). These appear to correlate best with the Fe–S_b modes at 289.2, 391.3, and 419.4 cm^{-1}, respectively, in the oxidized protein (Yachandra et al., 1983). The fact that only the bridging modes are strongly enhanced in the reduced protein implies that the S_t → Fe(III) CT band (near 520 nm in oxidized spinach ferredoxin) has been selectively diminished and that the principal S_b → Fe(III)

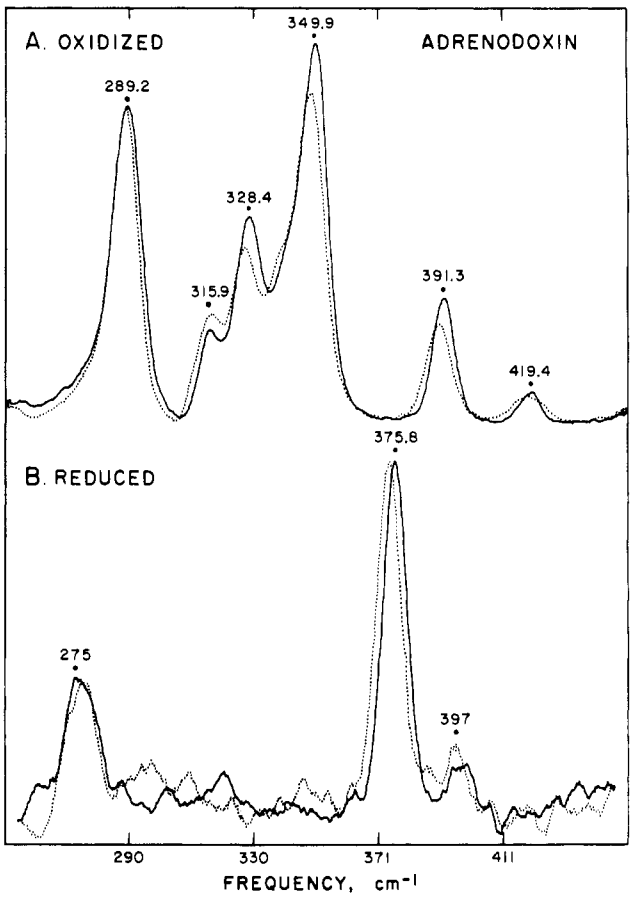

FIGURE 5: Resonance Raman spectra of bovine adrenodoxin in H_2O (—) and D_2O (···). Samples were 1.5 mM in 0.05 M Tris-HCl (pH 8.1 in H_2O, pH reading 8.3 in D_2O). Spectra of oxidized adrenodoxin were obtained at 15 K with 488.0-nm excitation (250 mW at the sample) with 6-cm^{-1} spectral resolution, 0.5 cm^{-1}/s scan rate, 10 scans, and a 13-point smooth. Spectra of reduced adrenodoxin were obtained on protein in a sealed capillary at 90 K with 530.9-nm excitation (70 mW at the samples) with 6-cm^{-1} spectral resolution, 0.5 cm^{-1}/s scan rate, 10 scans, and a 25-point smooth. Solvent subtraction and peak frequencies are described in Figure 4. Apparent peaks in the 290–330-cm^{-1} region are due to incomplete correction for the vibrational modes of the frozen solvent.

CT band (near 480 nm in oxidized spinach ferredoxin) has shifted to longer wavelength. Of the Fe–S_b modes, a pronounced deuterium shift of −1.8 cm^{-1} is observed for the peak at 375.8 cm^{-1}, while the peak at 275 cm^{-1} shows no detectable shift (Table I). The considerably larger −1.8-cm^{-1} shift of the Fe_2S_2 symmetric stretch in reduced adrenodoxin compared to the −1.1-cm^{-1} shift in oxidized adrenodoxin suggests that the hydrogen bonding of the sulfur ligands is stronger in the reduced form. These results are consistent with the finding from electron spin–echo measurements that hydrogens associated with the iron–sulfur center in adrenodoxin exchange considerably more slowly when the protein is in the reduced state (Orme-Johnson et al., 1983).

The resonance Raman spectrum of oxidized ferredoxin from *S. platensis* is shown in Figure 6. Despite the fact that this protein is of bacterial origin, its spectrum is close to that of spinach ferredoxin (Figure 4). A similar observation was made previously for the Raman spectrum of ferredoxin from *Halobacterium halobium* (Osaki et al., 1983), which has a conserved amino acid sequence in the region of the iron–sulfur cluster but is quite different from spinach ferredoxin elsewhere in the molecule. Since ferredoxin from *S. platensis* is a chloroplast-type protein, its amino acid sequence is more closely related to that of spinach ferredoxin (Wada et al.,

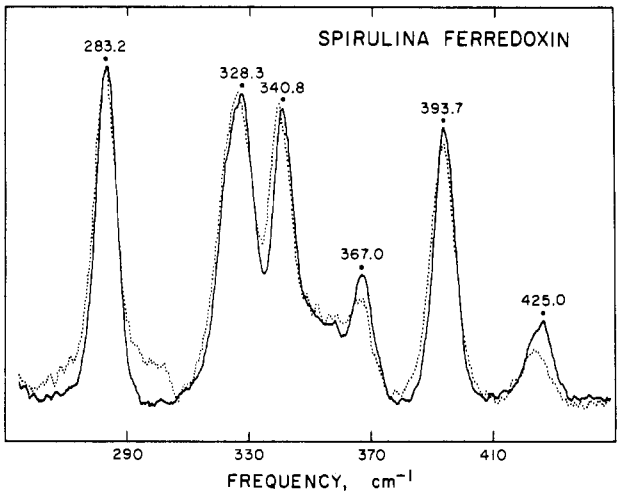

FIGURE 6: Resonance Raman spectra of oxidized ferredoxin from *Spirulina platensis* in H_2O (—) and D_2O (···). Samples were 3 mM in 0.05 M Tris-HCl (pH 8.0 in H_2O, pH reading 8.2 in D_2O). Spectral details as in Figure 4.

1975). Small differences between the Raman spectra of *S. platensis* ferredoxin and spinach ferredoxin occur in the Fe–S_t vibrations where the peaks at 328.3 and 340.8 cm^{-1} are better resolved, being separated by an additional 4 cm^{-1}, and a shoulder at 315 cm^{-1} is more apparent. Despite the stronger spectral similarity between *S. platensis* ferredoxin and spinach ferredoxin than adrenodoxin, the deuterium isotope effects in *S. platensis* ferredoxin (Figure 6, Table I) are less marked than those in the other two proteins. The Fe–S_b modes at 367.0 and 393.7 cm^{-1} shift by only −0.6 and −0.2 cm^{-1}, while the Fe–S_t modes at 328.3 and 340.8 cm^{-1} show shifts of −0.5 to −0.7 cm^{-1}. This pattern was not significantly altered by increasing the time of exposure of *S. platensis* ferredoxin from 4 days in D_2O to 14 days. The smaller deuterium dependence of the bridging modes is consistent with the X-ray crystallographic information (Figure 2), which indicates that the bridging sulfides are involved in fewer hydrogen bonds than the terminal thiolates. On the basis of the Raman data, it also seems probable that the bridging sulfides in spinach ferredoxin and adrenodoxin are more strongly hydrogen bonded than in *S. platensis* ferredoxin.

Discussion

The 0.3- to 2.0-cm^{-1} shifts in the Raman spectra of deuterium-substituted ferredoxin and adrenodoxin are similar to those observed in the Raman spectra of deuterium-substituted blue copper proteins. The only common structural feature in these proteins that is likely to result in deuterium sensitivity of metal-cluster vibrations is hydrogen bonding of the sulfur ligands to the protein backbone. Alternative possibilities such as contributions from imidazole or amide vibrational modes in the case of the blue copper proteins (Nestor et al., 1984; Blair et al., 1985) can be effectively ruled out as sources of deuterium dependence in the iron–sulfur proteins: the $Fe_2S_2(Cys)_4$-containing proteins are devoid of imidazole ligands, and the majority of the peaks in the Raman spectrum can be accounted for by the normal modes of the iron–sulfur cluster (Yachandra et al., 1983). The alternative explanation of a protein conformation change upon deuterium substitution seems unlikely for the following reasons: (1) A substantial conformational change would be required to account for the fact that all of the iron–sulfur vibrations are affected. (2) It is unlikely that a similar conformational change would occur in two such weakly related proteins as spinach ferredoxin and adrenodoxin, which differ considerably in their redox potentials and magnetic circular dichroism spectra (Johnson et al., 1982) as well as in their resonance Raman spectra. (3) Electron density maps of deuterium-exchanged crystalline proteins such as myoglobin and trypsin are essentially superimposable on those obtained in H_2O (Phillips & Schoenborn, 1981; Kossiakoff & Spencer, 1980). (4) Resonance Raman studies of the Fe–O–Fe site in hemerythrin showed that deuterium isotope effects on the Fe–O–Fe vibration were only observed in the presence of iron ligands capable of hydrogen-bond donation (e.g., hydroxide and hydroperoxide) and, thus, were not due to deuterium exchange elsewhere in the protein (Shiemke et al., 1986).

In all the Fe_2S_2 proteins investigated, deuterium shifts were observed for vibrations of both the bridging and the terminal iron–sulfur bonds. This is consistent with the known structure of *S. platensis* ferredoxin in which bridging as well as terminal sulfurs are hydrogen-bonded to the polypeptide backbone (Figure 2). The general expectation is that the absolute *frequency* of a stretching mode will decrease as the strength of the hydrogen bond increases. Since most of the deuterium isotope shifts in the ferredoxins and adrenodoxin are to lower energy, this implies that the deuterium-containing donors (e.g., ND and OD) are hydrogen bonding more strongly to the sulfur ligands than the corresponding hydrogen-containing donors (i.e., NH and OH). Such an interpretation is supported by the observation, particularly for spinach ferredoxin, that the 310–395-cm^{-1} peaks have also undergone decreases in intensity relative to the 285-cm^{-1} peak. Studies of the effect of hydrogen bonding on the Raman spectra of small organic molecules have shown that Raman *intensities* typically decrease with increasing hydrogen-bond strength (Hadži & Bratos, 1976).

On the basis of their amino acid sequence homology and resonance Raman spectra, the ferredoxins from *Spirulina platensis* and from spinach are expected to have quite similar iron–sulfur cluster configurations. Yet the Fe–S_t mode at 340.8 cm^{-1} as well as the Fe–S_b modes at 367.0 and 393.7 cm^{-1} in *S. platensis* ferredoxin shows considerably smaller deuterium shifts. It is likely that this reflects a diminished degree of hydrogen bonding in *S. platensis* ferredoxin. Such changes in the degree of hydrogen bonding can be due either to differences in the absolute strengths of particular hydrogen bonds or to differences in the total number of hydrogen bonds. Analysis of the amino acid sequences of these two proteins in the region of the iron–sulfur cluster shows that residues 40–50 are totally conserved with the exception of position 45, which is an alanine in *S. platensis* ferredoxin and a serine in spinach ferredoxin (Wada et al., 1975). Inspection of the protein conformation in the vicinity of alanine 45 in *S. platensis* ferredoxin by computer graphics suggests that a serine OH in this location could be within 4.1 Å of the Cys-41 sulfur atom or within 5.3 Å of one of the bridging sulfur atoms. If this serine were to be involved in such additional hydrogen bonds, it could explain the greater deuterium sensitivity of the Raman peaks in spinach ferredoxin. However, adrenodoxin, which has a similar deuterium dependence to spinach ferredoxin, is lacking the analogous serine at position 45 (Tanaka et al., 1973). However, it has a far lower degree of sequence homology, and its Raman spectrum is indicative of altered polypeptide conformations in the region around the iron–sulfur cluster (Yachandra et al., 1983). In this case, the increased hydrogen bonding could be due to a larger number of immobilized water molecules in the vicinity of the Fe-S cluster.

The Fe-S_b mode at 275–290 cm^{-1} in spinach ferredoxin and oxidized or reduced adrenodoxin shows a consistently smaller deuterium shift (0 to −0.3 cm^{-1}) than the Fe–S_b symmetric

stretch at 376–393 cm^{-1}, which decreases by –0.8 to –1.8 cm^{-1} in D_2O. Solely on the basis of the difference in their vibrational energies, the 275–290-cm^{-1} peak would have been expected to exhibit a deuterium frequency shift only 50% of that of the 376–393-cm^{-1} peak. However, the orthogonal disposition of the B_{1u} and A_{1g} vibrational displacements could account for the greater difference in their response to hydrogen-bonding effects. Although the 275–290-cm^{-1} peak also has an anomalously high Raman intensity for a B_{1u} mode, this has been ascribed to a distortion of the iron–sulfur cluster away from D_{2h} symmetry, possibly due to the prevalence of hydrogen bonds on one end of the cluster (Yachandra et al., 1983). An alternative explanation is that the ~285-cm^{-1} peak includes a contribution from the terminal Fe–S–C bend, particularly since an analogous Cu–S–C bend is observed near 260 cm^{-1} in Raman spectra of blue copper proteins (Kuila et al., 1987; Blair et al., 1985). If the ~285-cm^{-1} peak does represent an admixture of Fe–S_b stretching and Fe–S_t–C bending modes, this could explain its lesser sensitivity to deuterium exchange, both in frequency shift and in relative peak intensity.

The Fe–S_b mode at ~425 cm^{-1} has the most unusual behavior in D_2O; its extensive broadening indicates a splitting into higher and lower energy components. From comparisons with model compounds it has been noted that this vibration is also anomalously intense for D_{2h} symmetry (Yachandra et al., 1983). As a result, this vibrational feature has been suggested to contain some terminal cysteine S–C–C bending character, again by analogy to the spectra of the blue copper proteins (Kuila et al., 1987; Blair et al., 1985). It has been observed empirically that the frequency of bending vibrations typically increases with the extent of hydrogen bonding of the terminal atoms (Hadži & Bratos, 1976). Thus, the deuterium-dependent shift of a component of the ~425-cm^{-1} peak to higher energy in spinach ferredoxin and adrenodoxin, as well as analogous positive shifts at 380 and 419 cm^{-1} in copper-substituted alcohol dehydrogenase (+NADH) in D_2O (Maret et al., 1986), could signal a more significant δ(S–C–C) contribution to these vibrational modes.

In comparing the resonance Raman spectra of the chloroplast-type ferredoxins from spinach and *S. platensis* with that of adrenodoxin, it appears that the principal variations occur in the frequencies and intensities of the Fe–S_t modes between 310 and 350 cm^{-1}. Yachandra et al. (1983) have proposed that these differences may relate to the conformations of the cysteine ligands in each protein. This is likely to have considerable bearing on the differences in redox potentials, which are –420 and –390 mV for spinach and *S. platensis* ferredoxin, respectively, versus –270 mV for adrenodoxin (Thompson, 1985). In contrast, on the basis of deuterium isotope effects, the hydrogen-bonding networks of spinach ferredoxin and adrenodoxin appear to be more similar to one another than to that of *S. platensis* ferredoxin. Although differential degrees of hydrogen bonding have been proposed to play a role in modulating the redox potential of iron–sulfur proteins, particularly those with Fe_4S_4 clusters (Adman et al., 1975; Sheridan et al., 1981), it is clear that additional factors such as hydrophobic interactions are important, as well (Krishnamoorthi et al., 1986; Ueyama et al.,1985). If the deuterium shifts in the Raman spectra of the Fe_2S_2-containing proteins are an adequate measure of hydrogen-bond strengths, then it is likely that the observed differences in redox potential between the plant-type ferredoxins and adrenodoxin are not primarily due to differences in the degree of hydrogen bonding.

Conclusions

The observation of deuterium-induced changes in the frequency and intensity of vibrational modes has long been of use in detecting the occurrence of hydrogen bonds and in quantitating hydrogen-bond strengths in organic and inorganic compounds (Hadži & Bratos, 1976; Joesten & Schaad, 1974). The present work indicates that such effects are also prevalent in the resonance Raman spectra of metal–sulfur-containing proteins and are diagnostic of the hydrogen bonding of sulfur ligands to the protein donors. Comparison of our results on spinach ferredoxin and bovine adrenodoxin, for which no X-ray structural information yet exists, indicates that their iron–sulfur clusters are as extensively hydrogen bonded as the sulfur atoms in *S. platensis* ferredoxin and perhaps even more so.

Extrapolation of these findings to the resonance Raman spectra of the blue copper proteins leads to the conclusion that the ubiquitous deuterium shifts of the high-frequency modes (330–450 cm^{-1}) can be explained by hydrogen bonding of the cysteine thiolate ligand, without the need to invoke contributions from histidine ligand modes. Hence, the observation of deuterium isotope effects in M–L stretching vibrations does not necessarily imply imidazole ligation. Deuterium shifts of peaks in the 240–300-cm^{-1} region, however, are more likely to be diagnostic of imidazole ligation because Cu–N(imidazole) stretching vibrations do occur in this energy range (Cornilsen & Nakamoto, 1974; Larrabee & Spiro, 1980).

The magnitude of the deuterium shift in the characteristic Fe–S stretching modes of ferredoxin and adrenodoxin varies from 0.3 to 2.0 cm^{-1}. The deuterium shifts for the Cu–S modes in the blue copper proteins are in this same range. In an earlier study of the oxo-bridged binuclear iron protein hemerythrin, the symmetric Fe–O–Fe vibration was found to undergo a 4-cm^{-1} shift upon deuterium substitution (Shiemke et al., 1986). The larger deuterium shift for an oxygen-containing ligand is consistent with the fact that hydrogen bonds to oxygen exhibit $-\Delta H°$ values of 2–5 kcal/mol (Pimentel & McClellan, 1971; Murthy & Rao, 1968) and, consequently, are considerably stronger than hydrogen bonds to sulfur with corresponding $-\Delta H°$ values of 0.5–2 kcal/mol (Pogorely, 1977).

The tendency of metal ligands to undergo hydrogen bonding in metalloproteins could be mainly a consequence of the need to dissipate charge density in a nonpolar environment. However, it is likely that protein structures have evolved to optimize such hydrogen bonding and thereby create metal clusters with more useful properties. In the blue copper proteins, for example, hydrogen bonding may well contribute to the protein-imposed geometry at the copper site. In the crystal structure of azurin from *A. denitrificans* it has been noted that the atoms in the vicinity of the metal center have smaller thermal parameters, indicating a lowered mobility in this part of the molecule. In keeping with this finding is the observation that all three of the primary copper ligands are hydrogen-bonded: Cys-112 to the amide NH's of residues 47 and 114, His-46 to the amide carbonyl of residue 10, and His-117 to an immobilized water molecule (E. N. Baker, personal communication). A similar increase in rigidity in the region surrounding the metal cluster has been proposed for the $Fe_2S_2(Cys)_4$-containing ferredoxins (Hearshen et al., 1986). In the case of the $Fe_4S_4(Cys)_4$-containing proteins, the degree of hydrogen bonding may well affect the electrostatic character of the iron-sulfur site and, thus, be an important factor in regulating the redox potential (Sheridan, 1981). Resonance Raman studies of Fe_4S_4 clusters and their deuterium isotope effects are in progress and will be reported elsewhere.

Acknowledgments

We thank Dr. Edward N. Baker for pointing out the importance of hydrogen bonds in metal–sulfur clusters, Dr. Bruce

Branchaud for making us aware of the difference between H···S and H···O bond strengths, and Dr. John H. Golbeck for advising us in the purification of ferredoxin. We are also grateful to Dr. Ronald E. Stenkamp for helping us with the computer graphics analysis.

Registry No. Cys, 52-90-4; D_2, 7782-39-0.

REFERENCES

Adman, E. T. (1985) in *Metalloproteins* (Harrison, P., Ed.) Part 1, pp 1–42, Verlag Chemie, Weinheim, FRG.

Adman, E., Watenpaugh, K. D., & Jensen, L. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4854–4858.

Ainscough, E. W., Bingham, A. G., Brodie, A. M., Ellis, W. R., Gray, H. B., Loehr, T. M., Plowman, J. E., Norris, G. E., & Baker, E. N. (1987) *Biochemistry 26*, 71–82.

Baker, E. N., & Hubbard, R. E. (1984) *Prog. Biophys. Mol. Biol. 44*, 97–179.

Beardwood, P., & Gibson, J. F. (1984) *J. Chem. Soc., Dalton Trans.*, 1507–1516.

Blair, D. F., Campbell, G. W., Schoonover, J. R., Chan, S. I., Gray, H. B., Malmström, B. G., Pecht, I., Swanson, B. I., Woodruff, W. H., Cho, W. K., English, A. M., Fry, H. A., Lum, V., & Norton, K. A. (1985) *J. Am. Chem. Soc. 107*, 5755–5766.

Buckingham, A. D., & Fan-Chen, L. (1981) *Int. Rev. Phys. Chem. 1*, 253–269.

Carter, C. W., Jr., Kraut, J., Freer, S. T., & Alden, R. A. (1974) *J. Biol. Chem. 249*, 6339–6346.

Church, W. B., Guss, J. M., Potter, J. J., & Freeman, H. C. (1986) *J. Biol. Chem. 261*, 234–237.

Cornilsen, B. C., & Nakamoto, K. (1974) *J. Inorg. Nucl. Chem. 36*, 2467–2471.

Eklund, H., & Brändén, C.-I. (1983) in *Zinc Enzymes* (Spiro, T. G., Ed.) pp 124–152, Wiley, New York.

Garrett, T. P. J., Clingeleffer, D. J., Guss, J. M., Rogers, S. J., & Freeman, H. C. (1984) *J. Biol. Chem. 259*, 2822–2825.

Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521–563.

Hadži, D., & Bratos, S. (1976) in *The Hydrogen Bond* (Schuster, P., Zundel, G., & Sandorfy, C., Eds.) pp 565–611, North-Holland, Amsterdam.

Hearshen, D. O., Hagen, W. R., Sands, R. H., Grande, H. J., Crespi, H. L., Gunsalus, I. C., & Dunham, W. R. (1986) *J. Magn. Reson. 69*, 440–459.

Joesten, M. D., & Schaad, L. J. (1974) in *Hydrogen Bonding*, pp 2–15, Dekker, New York.

Johnson, M. K., Spiro, T. G., & Mortenson, L. E. (1982) *J. Biol. Chem. 257*, 2447–2452.

Kossiakoff, A. A., & Spencer, S. A. (1980) *Nature (London) 288*, 414–416.

Krishnamoorthi, R., Markley, J. L., Cusanovich, M. A., Przysiecki, C., & Meyer, T. E. (1986) *Biochemistry 25*, 60–67.

Kuila, D., Fee, J. A., Schoonover, J. R., Woodruff, W. H., Batie, C. J., & Ballou, D. P. (1987) *J. Am. Chem. Soc. 109*, 1559–1561.

Larrabee, J. A., & Spiro, T. G. (1980) *J. Am. Chem. Soc. 102*, 4217–4223.

Loehr, T. M., Keyes, W. E., & Pincus, P. A. (1979) *Anal. Biochem. 96*, 456–463.

Magliozzi, R. S., McIntosh, B. A., & Sweeney, W. V. (1982) *J. Biol. Chem. 257*, 3506–3509.

Maret, W., Shiemke, A. K., Wheeler, W. D., Loehr, T. M., & Sanders-Loehr, J. (1986) *J. Am. Chem. Soc. 108*, 6351–6359.

Meyer, J., Moulis, J.-M., & Lutz, M. (1986) *Biochim. Biophys. Acta 873*, 108–118.

Murthy, A. S. N., & Rao, C. N. R. (1968) *Appl. Spectrosc. Rev. 2*, 69–191.

Musci, G., Desideri, A., Morpurgo, L., & Tosi, L. (1985) *J. Inorg. Biochem. 23*, 93–102.

Nestor, L., Larrabee, J. A., Woolery, G., Reinhammar, B., & Spiro, T. G. (1984) *Biochemistry 23*, 1084–1093.

Norris, G. E., Anderson, B. F., & Baker, E. N. (1986) *J. Am. Chem. Soc. 108*, 2784–2785.

Orme-Johnson, W. H., & Beinert, H. (1969) *J. Biol. Chem. 244*, 6143–6148.

Orme-Johnson, N. R., Mims, W. B., Orme-Johnson, W. H., Bartsch, R. G., Cusanovich, M. A., & Peisach, J. (1983) *Biochim. Biophys. Acta 748*, 68–72.

Ozaki, Y., Nagayama, K., Kyogoku, Y., Hase, T., & Matsubara, H. (1983) *FEBS Lett. 152*, 236–240.

Phillips, S. E. V., & Schoenborn, B. P. (1981) *Nature (London) 292*, 81–82.

Pimentel, G. C., & McClellan, A. L. (1971) *Annu. Rev. Phys. Chem. 22*, 347–385.

Pogorely, V. K. (1977) *Russ. Chem. Rev. (Engl. Transl.) 46*, 316–336.

Schneider, G., Eklund, H., Cedergren-Zeppezauer, E., & Zeppezauer, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5289–5293.

Schneider, G., Cedergren-Zeppezauer, E., Eklund, H., Knight, S., & Zeppezauer, M. (1985) *Biochemistry 24*, 7503–7510.

Sheridan, R. P., Allen, L. C., & Carter, C. W., Jr. (1981) *J. Biol. Chem. 256*, 5052–5057.

Shiemke, A. K., Loehr, T. M., & Sanders-Loehr, J. (1986) *J. Am. Chem. Soc. 108*, 2437–2443.

Sjöberg, B.-M., Loehr, T. M., & Sanders-Loehr, J. (1982) *Biochemistry 21*, 96–102.

Solomon, E. I., Penfield, K. W., & Wilcox, D. E. (1983) *Struct. Bonding (Berlin) 53*, 1–57.

Tanaka, M., Haniu, M., Yasunobu, K. T., & Kimura, T. (1973) *J. Biol. Chem. 248*, 1141–1157.

Thompson, A. J. (1985) in *Metalloproteins* (Harrison, P., Ed.) Part 1, pp 79–120, Verlag Chemie, Weinheim, FRG.

Tsukihara, T., Fukuyama, K., Nakamura, M., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., Hase, T., & Matsubara, H. (1981) *J. Biochem. (Tokyo) 90*, 1763–1773.

Tsukihara, T., Fukuyama, K., & Katsube, Y. (1986) in *Iron-Sulfur Protein Research* (Matsubara, H., Katsube, Y., & Wada, K., Eds.) pp 59–69, Springer-Verlag, West Berlin.

Ueyama, N., Kajiwara, A., Terakawa, T., Ueno, S., & Nakamura, A. (1985) *Inorg. Chem. 24*, 4700–4704.

Wada, K., Hase, T., Tokunaga, H., & Matsubara, H. (1975) *FEBS Lett. 55*, 102–104.

Woodruff, W. H., Dyer, R. B., & Schoonover, J. R. (1987) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. III, Wiley, New York (in press).

Yachandra, V. K., Hare, J., Gewirth, A., Czernuszewicz, R. S., Kimura, T., Holm, R. H., & Spiro, T. G. (1983) *J. Am. Chem. Soc. 105*, 6462–6468.