Detailing the conformational switching that accompanies biological assembly is an important problem in developmental biology. We have demonstrated in this paper that Raman spectroscopy can be a sensitive probe of conformational changes affecting macromolecular assembly. Raman spectroscopy would appear to be the method of choice for monitoring conformational switching in these capsid assemblies since particle size and scattering artifacts severely limit the use of other spectroscopic probes, such as NMR and CD spectroscopies.

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^{1}H NMR Studies of Oxidized High-Potential Iron Protein from *Chromatium vinosum*. Nuclear Overhauser Effect Measurements†

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ABSTRACT: ^{1}H nuclear Overhauser effect experiments on the isotropically shifted signals of oxidized *Chromatium vinosum* HiPIP have been used to identify the four β-CH_2 geminal couples of the cysteine ligands. A partial assignment to individual residues has been proposed from a computer graphics analysis of the X-ray structure. Tentative assignments of other resonances are discussed.

High-potential iron proteins (HiPIP's)[1] are a class of redox proteins containing a $[Fe_4S_4]$ cluster that is bound by four cysteine residues (Carter et al., 1974). HiPIP's have been isolated from a number of bacterial sources and are characterized by unusually high reduction potentials ($E^{\circ\prime} \sim 0.35$ V) that are significantly different from those of related low-potential ferredoxins ($E^{\circ\prime} \sim -0.4$ V), which contain one or two clusters that are structurally identical with the former (Palmer, 1975; Carter, 1977). HiPIP's have been the subject of a variety of spectroscopic studies (Dus et al., 1967, 1973); however, ^{1}H NMR is of particular value for mapping the region around a paramagnetic prosthetic group (Nettesheim et al., 1983; Krishnamoorthi et al., 1986, 1989; Moss et al., 1969; Holm et al., 1974; Que et al., 1974; Reynolds et al.,

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[1] Abbreviations: HiPIP, high-potential iron protein; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; WEFT, water-eliminated Fourier transform; MODEFT, modified driven equilibrium Fourier transform.

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1978; Sola et al., 1989a,b). The protein from the photosynthetic bacterium *Chromatium vinosum* is of low molecular weight and can be readily studied. Hyperfine-shifted signals have been observed in both oxidized and reduced states (Nettesheim et al., 1983; Krishnamoorthi et al., 1986, 1989; Sola et al., 1989a,b): the reduced protein shows a residual paramagnetism at room temperature as a result of a partial quenching of the antiferromagnetic coupling among the iron atoms, while the oxidized state is paramagnetic with a magnetic susceptibility corresponding to one unpaired electron (Moss et al., 1969). The hyperfine-shifted signals therefore fall in a narrow region (9–17 ppm) in the former case, while they are spread over a much wider chemical shift range in the latter (100 to −40 ppm). In the *C. vinosum* protein, the hyperfine-shifted resonances are sensitive to the ionization of His-42 (Nettesheim et al., 1983), while in the *Chromatium gracile* analogue they feel the ionization of two residues near the cluster (Glu-44 and His-42) (Sola et al., 1989a,b). The electronic properties of the cluster are therefore sensitive to the acid–base equilibria of nearby residues. A reliable assignment of these resonances is required to understand the role of the polypeptide backbone in determining the electronic and magnetic properties of the cluster. Furthermore, the rationalization of the shift pattern is a necessary preliminary step toward monitoring structural changes during the interaction of such proteins with biological redox partners. Some tentative assignments of the hyperfine-shifted signals have been proposed previously on the basis of their chemical shifts, relaxation properties, temperature, and pH dependencies (Krishnamoorthi et al., 1986, 1989; Sola et al., 1989a,b). ^{1}H NOE experiments on paramagnetic proteins were first reported in 1979 (Trewhella et al., 1979) and have become a valuable tool for spectral assignments, on heme proteins in particular (Keller, 1980; Gordon & Wuthrich, 1978; Unger et al., 1985; Pande et al., 1986; Lecomte et al., 1985). Recently, with medium-field spectrometers (200–300 MHz), suitable pulse sequences, and reasonable acquisition times, NOE connectivities among the hyperfine-shifted signals corresponding to the β-CH_2 geminal couples of the cysteines binding the metal cluster have been detected for reduced [Fe_2S_2] proteins (Bertini et al., 1990) and for oxidized 2[Fe_4S_4] ferredoxins with the cluster in the divalent oxidation state (ground state S = O) (Bertini et al., 1989). We have now carried out a ^{1}H NOE investigation on the oxidized HiPIP from *C. vinosum* that contains a [Fe_4S_4] cluster in the trivalent state (ground state $S = {}^1/_2$) and have reevaluated previous spectral assignments for this and related proteins (Krishnamoorthi et al., 1986, 1989; Sola et al., 1989a,b).

Materials and Methods

Chromatium vinosum [ATCC 17899 (strain D)] was grown, and HiPIP isolated, according to the procedures of Bartsch (1978). ^{1}H NMR measurements were carried out on ca. 5 mM protein samples. The oxidized protein was obtained by the addition of a trace of potassium ferricyanide. ^{1}H NMR spectra were recorded at 300 MHz on Bruker MSL and Bruker CXP spectrometers, using 16K data points over a 72-kHz bandwidth. The signal from residual water was suppressed by using the WEFT pulse sequence (180–τ–90–AQ+delay) (Inubushi & Becker, 1983) with recycle times of 70–100 ms and delay times (τ) of 50–100 ms. Chemical shifts are reported in ppm by assigning a 4.8 ppm value to the residual water peak. NOE measurements were performed by using the same sequence with a selective pulse from the decoupler of 0.01–0.02 W that was maintained for nine-tenths of the delay time τ. Care was taken in the choice of saturation power in order to avoid nonselective effects. Difference spectra were obtained directly by cycling the decoupler frequency according to the following scheme: $\omega_2 - \omega_2 + \delta - \omega_2 - \omega_2 - \delta$, where ω_2 is the frequency of the irradiated signal and δ the off-resonance offset. Typical spectra were obtained with 20–25 blocks of 16 000 scans each. A line broadening of 25 Hz was used throughout. Each spectrum was repeated to verify the results. With these experimental conditions, the detectability limit for the NOE is estimated at 0.5%. T_1 values that had been previously obtained (Sola et al., 1989a,b) were checked with the MODEFT sequence (Hockmann & Kellerhals, 1980).

A computer graphics analysis of the environment of the [Fe_4S_4] cluster was performed with the Biograf molecular graphics/mechanics program [graphics analysis was performed with Biograf, version 1.50 (Biodesign Inc., Pasadena, CA)] on an Evans & Sutherland terminal interfaced with a VAX 11/750 computer. The atomic coordinates of the protein were from the X-ray structure at 2-Å resolution (Carter et al., 1974a,b).

Theoretical Aspects

The change in the intensity of the NMR resonance of a given spin, when that of a dipole-coupled spin belonging to the same molecule is saturated, is known as the nuclear Overhauser effect (Noggle & Shirmer, 1971). For an isolated two-spin system (ij) separated by a distance r_{ij} and tumbling with a correlation time τ_c, the spectral density functions that regulate the transition probabilities among the energy levels are related to the former geometric and motional parameters. In particular, in the high-field case ($\omega^2\tau_c^2 \gg 1$), the cross-relaxation rate is given by

$$\sigma_{ij} = -\hbar^2\gamma^4\tau_c/10r_{ij}^6 \quad (1)$$

In the absence of local motions, τ_c coincides with the protein tumbling time. If a saturating frequency is applied selectively to j, the NOE on i develops as a function of the time of irradiation according to eq 2 where ρ_i is the intrinsic spin–

$$\eta_{ij} = \sigma_{ij}(1 - e^{-\rho_i t})/\rho_i \quad (2)$$

lattice relaxation rate of i. The cross-relaxation term allows the internuclear distance to be estimated from NOE measurements. For long irradiation times ($t \gg \rho_i^{-1}$), eq 2 gives

$$\eta_{ij} = \sigma_{ij}/\rho_i \quad (3)$$

which is the maximum NOE effect observable and is known as the "steady-state NOE".

In paramagnetic systems, the large ρ_i values reduce the intensity of the observed NOE's. As a result, a large signal-to-noise ratio is required to detect NOE's that may be lower than 1%. Such NOE's are all primary, however, since spin diffusion is quenched.

Results

The ^{1}H NMR spectra of oxidized HiPIP from both *C. vinosum* and several other bacterial sources have been reported previously (Nettesheim et al., 1983; Krishnamoorthi et al., 1986, 1989; Sola et al., 1989a,b). In the former, seven hyperfine-shifted signals appear downfield of the diamagnetic region and two upfield (Figure 1, top; Table I). These signals must arise from the β-CH_2 protons of the cysteine ligands and possibly from other protons adjacent to the cluster. No signals corresponding to exchangeable protons have been detected by recording the spectra in H_2O. The internuclear distance between two geminal β-CH_2 protons (about 1.7 Å) is small enough to allow a strong reciprocal NOE effect that has re-

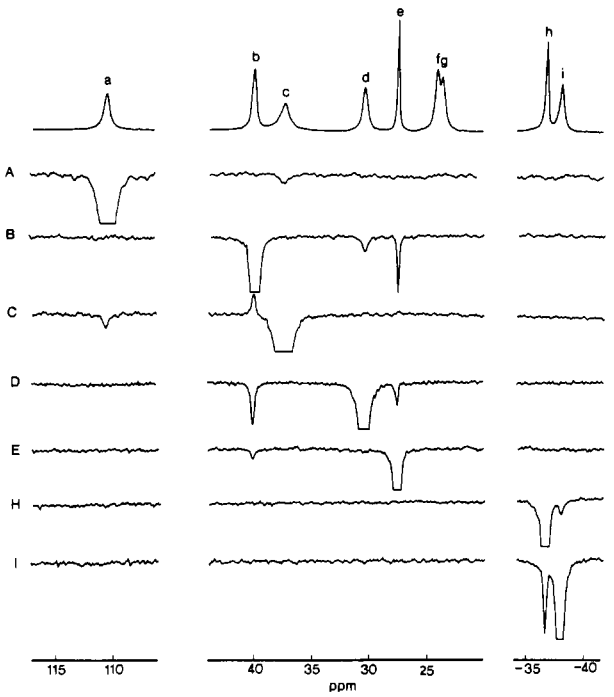

FIGURE 1: (Top) Hyperfine-shifted signals in the 300-MHz ^{1}H NMR spectrum of oxidized *C. vinosum* HiPIP obtained in μ = 0.1 M sodium phosphate buffer in D_2O, pH 7.0, 296 K. Traces from A to I show steady-state NOE difference spectra obtained by saturating the signal corresponding to the letter.

Table I: NMR Parameters for the Hyperfine-Shifted Signals in the Oxidized HiPIP from *Chromatium vinosum*[a]

signal	δ (ppm)	T_1 (ms)[b]
a	110.60	5.5
b	40.10	14.4
c	37.46	3.9
d	30.40	6.3
e	27.52	43.0
f	24.15	10.9
g	23.70	8.9
h	-35.57	25.0
i	-37.87	8.6
l	10.03	57.7
m	7.64	86.5

[a] Sodium phosphate buffer (μ = 0.1 M, pH 7.0, 298 K). [b] Estimated with the MODEFT sequence (errors are within 10%).

cently been detected in $[Fe_2S_2]$ and $[Fe_4S_4]$ ferredoxins (Bertini et al., 1989, 1990). A previous unsuccessful attempt to detect NOE's among the hyperfine-shifted signals of HiPIP's from *Ectothiorhodospira halophila* and *Ectothiorhodospira vacuolata* has been noted (Krishnamoorthi et al., 1986). In this paper, we report a number of proton connectivities that have been detected in the *C. vinosum* protein. The difference spectra obtained upon saturating in turn all nine resonances (each of which integrate to one proton) are shown in Figures 1 and 2, and a map of the NOE connectivities is reported in Table II. The interproton distances were evaluated from eq 3 using a τ_c value of 3.3×10^{-9} s obtained from the Debye relationship. The following cysteine β-CH_2 pairs can be straightforwardly assigned: a–c, b–d, and h–i. In all cases, the NOE's of each pair give interproton distances in the range 1.6–1.8 Å. The two signals f and g are very close together, and their separation does not increase at different temperatures (Sola et al., 1989a,b; Phillips et al., 1970). Even with very low decoupler powers and different values of the decoupler off-resonance offset, it was not possible to discriminate between a true NOE and an effect due to a nonselective saturation.

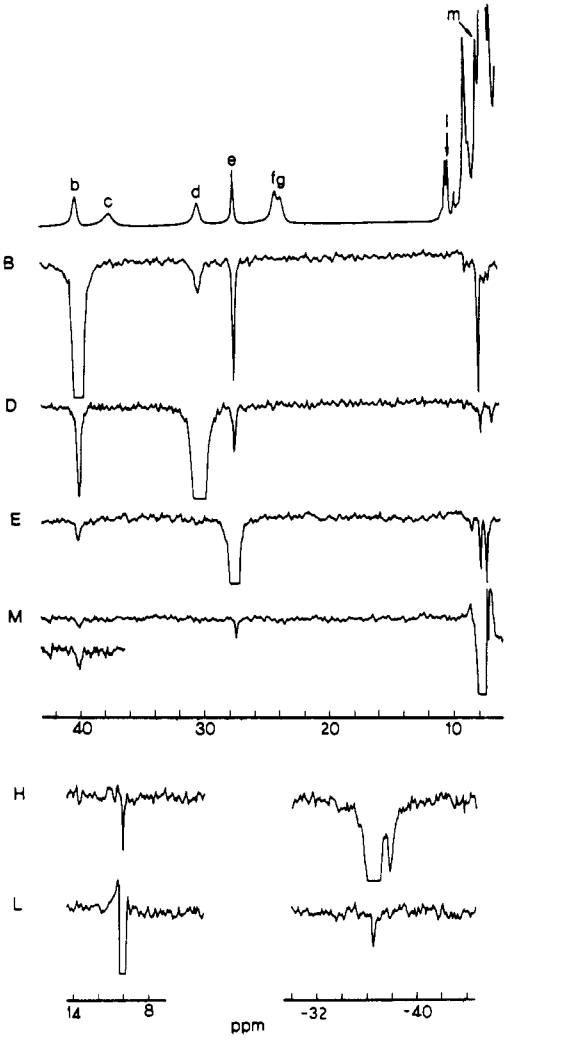

FIGURE 2: ^{1}H steady-state NOE difference spectra of isotropically shifted signals and resonances in the diamagnetic region. Letters A–L correspond to the irradiated peak. All spectra were obtained in μ = 0.1 M sodium phosphate buffer in D_2O, pH 7.0, 296 K.

Table II: Nuclear Overhauser Effects among the Hyperfine-Shifted Signals in the Oxidized Form of HiPIP from *Chromatium vinosum*[a,b]

irradiated peaks	observed peaks								
	a	b	c	d	e	h	i	l	m
a			5.2						
b				5.0	7.2				6.3
c	7.0								
d		12.2			3.8				2.1
e		2.4							3.0
h							5.5	2.1	
i						16.9		2.0	
l						2.6			
m		1.3			1.4				

[a] The data are reported as a percent decrease of the signal intensity. [b] Spectra were obtained by using the conditions described in the footnotes of Table I.

However, since both resonances show anti-Curie behavior, while all the other signals follow the Curie law (Sola et al., 1989a,b; Phillips et al., 1970), and since saturation of the composite peak does not give rise to any NOE outside of the diamagnetic region, we can reasonably assign these resonances to the fourth, and last, geminal couple.

The sharp resonance e shows NOE connectivities with both signals of the cysteine β-CH_2 couple b,d, and the reverse effect is observed for the slower relaxing resonance b (Figures 1 and 2B,D,E). The estimated distances (2.2 and 2.5 Å) indicate

that signal e may correspond to the α-CH proton of the same cysteine residue. Computer graphics analysis of the X-ray structure of the protein shows that the α-CH proton of Cys-43 is oriented toward the cluster (the closest contact being 3.1 Å form the cysteine-bound iron atom), so the sizeable isotropic shift may be due to a dipolar contribution. All the other α-CH protons appear to be more distant from the cluster and would not be expected to show large shifts; the α-CH protons assigned so far in Fe_4S_4 proteins (Bertini et al., 1989) and $[Fe_2S_2]$ ferredoxins (Bertini et al., 1990) fall near or inside the diamagnetic region. The crystal structure of HiPIP also shows the Cys-43 α-CH proton at 2.5 and 2.8 Å from the methylene protons of the Cys-46 residue; however, signal e does not show any NOE with another β-CH_2 couple. It is worth noting that a ring proton of Tyr-19 (meta to the hydroxyl) experiences very close contact with the cluster (3.15 Å from an inorganic sulfur atom) and it could be a candidate for the assignment of e. Computer graphics indicates that it lies 2.1 and 3.5 Å from the β-CH_2 protons of Cys-77, although these distances are not fully consistent with the observed NOE connectivities. We cannot at this moment, however, exclude this possibility. No other hydrogen atoms appear as close to both the cluster and the β-CH_2 group of a cysteine residue. Signal e also shows an NOE with signal m (7.64 ppm) (Figure 2E,M), which in turn connects with both signals of the geminal couple b–d (Figure 2B,D). If one assigned signal e to the Cys-43 α-CH proton, signal m could correspond to the nonexchangeable NH proton (not exposed to solvent) of the same residue that lies 2.9 Å from the α-CH proton and 2.7 and 3.5 Å from the β-CH_2 geminal couple, in good agreement with the NOE distances. If we consider signal e as the Tyr-19 ring proton, the assignment of signal m becomes much more uncertain, and any further hypotheses would be purely speculative. A further signal (at 7.1 ppm) connects with signal e (Figure 2E), but we have too few data to offer specific assignments. We have observed that signals h and i, which correspond to a cysteine β-CH_2 pair, connect with a sharp resonance at 10.03 ppm (signal l) while the reverse effect is observed on the slower relaxing resonance h (Figure 2H,L). The estimated distance of 2.4 Å, and the chemical shift value, indicates that signal l may correspond to the α-CH proton of the same cysteine residue. No other significant and/or reproducible NOE's that could have been assigned to α-CH protons have been detected between the geminal couples and signals in the diamagnetic region.

Discussion

Of the nine hyperfine-shifted resonances that appear in the spectrum of the oxidized form, we have demonstrated that eight arise from the four β-CH_2 geminal pairs of the cysteine ligands. This has resulted in a reevaluation of the assignment of resonances previously attributed to other residues adjacent to the cluster (Krishnamoorthi et al., 1986, 1989; Sola et al., 1989a,b). It is worth noting that the two resonances of the a–c couple show dramatically different chemical shifts, while the difference in shielding of the components of the other pairs is much less pronounced. In particular, the signals of the h–i couple are very close together, and those of the f–g couple are almost overlapped. The contact shifts of the geminal β-CH_2 protons are expected to be related to the relative dihedral angles Fe–S–C–H (ϕ) of the iron-coordinated cysteine according to the relationship (Bertini & Luchinat, 1986; Poe et al., 1970; Stone & Maki, 1962):

$$A = \beta + B_2 \cos^2 \phi$$

where A is the Fermi contact coupling constant, $B_2 \cos^2 \phi$ describes the spin density transferred from the p orbital of the cysteine sulfur atom to the β-CH_2 protons through a hyperconjugative mechanism, and β is related to the spin density transferred to the protons by other mechanisms. This equation was obtained for, and has been principally applied to, simple Ni–amine complexes and alkyl radicals (Stone & Maki, 1986; Ho & Reilley, 1964; Fitzgerald & Drago, 1968; Zamarov et al., 1966) in which spin density is transferred to the protons predominantly by a σ-spin delocalization mechanism. Since the additional β term is in general neglected, the ratio of the $\cos^2 \phi$ values should be related to the ratio of the hyperfine shifts of the geminal CH_2 protons. The values of the dihedral angles for the cysteine residues were obtained from a computer graphics analysis of the crystallographic structure of the protein. Analysis of the $\cos^2 \phi$ ratios indicates that Cys-46 and Cys-63 are the most likely candidates to show a sizeable difference of chemical shift between the geminal CH_2 protons. These data do not allow individual assignments since the ratios between the $\cos^2 \phi$ from the X-ray structure and those between the chemical shifts of the various geminal protons do not appear to be directly comparable as absolute values, and can be handled only in a qualitative way. This may be due to the delocalization of unpaired spin onto the cysteine ligands. The negative chemical shift value of the geminal β-CH_2 couple h–i and the heterogeneous temperature behavior of the β-CH_2 proton signals must derive from the complex electronic structure of the oxidized Fe_4S_4 cluster. A recent theoretical study of magnetic coupling in $[Fe_4S_4]^{3+}$ clusters that describes the isotropic shift of the bound cysteine β-CH_2 protons in terms of contact coupling has helped to explain the presence of both upfield and downfield signals (Banci et al., 1990). However, the opposite sign of the isotropic shift for the α-CH proton proximal to the β-CH_2 couple h–i cannot be readily explained. A dipolar contribution to the shift cannot be excluded as a possible origin of this inversion.

A number of tentative assignments have previously been proposed for the homologous HiPIP from *C. gracile* (Sola et al., 1989a,b) and for other HiPIP's from various bacterial sources (Krishnamoorthi et al., 1986, 1989). Such assignments were performed by analyzing the magnitude of the hyperfine shift, the relaxation parameters, and the temperature and pH dependence of the signals. The NOE data described in this paper allow us to improve upon these previous assignments. In particular, the hypothesis that only one of the protons of the β-CH_2 pair is isotropically shifted, while the other probably falls inside the diamagnetic region due to an unfavorable value of the dihedral angle (ϕ) (Krishnamoorthi et al., 1986), can now be discounted. Furthermore, for both the *C. gracile* (Sola et al., 1989a,b) and *Rhodospirillum tenue* (Krishnamoorthi et al., 1989) proteins, it was correctly proposed that all the downfield-shifted paramagnetic signals other than signal e should correspond to β-CH_2 protons; nevertheless, the proposed individual assignments appear to be incorrect (Sola et al., 1989a,b). Likewise, the assignment of various hyperfine-shifted signals in the downfield region of the spectra of HiPIP's to protons belonging to aromatic residues (Krishnamoorthi et al., 1986) should be reexamined. Those appearing in the upfield region were tentatively assigned to a proton from an aromatic residue (most probably Tyr-19) and to a Cys α-CH proton (Krishnamoorthi et al., 1986, 1989; Sola et al., 1989a,b), but they actually are the components of a β-CH_2 couple.

These NOE experiments indicate that the hyperfine-shifted signals observed in the ^1H NMR spectrum of both the reduced and oxidized states of HiPIP from *C. vinosum* arise mostly

from the β-CH_2 groups of the cysteine ligands. These results also indicate that temperature and pH dependences, magnitude and sign of the chemical shift, and T_1 values must be used with care for the purposes of resonance assignments in these systems, if no other independent source of information is available.

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