Proton Magnetic Resonance Studies of Chromatium High-Potential Iron Protein

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Abstract. Contact-shifted nuclear magnetic resonances, arising from molecular paramagnetism, have been observed in both reduced and oxidized forms of the high-potential iron protein (HiPIP) isolated from Chromatium. Contact shifts of the reduced, formally diamagnetic form increase with temperature, indicating antiferromagnetic exchange coupling of the component iron atoms with thermal population of a magnetic state. In the oxidized form of HiPIP (formally $S = \frac{1}{2}$), contact-shifted resonances attributed to the $\beta$-CH$_3$ groups of two cysteine residues display approximate Curie law behavior, while contact-shifted resonances assigned to the two other cysteine residues continue to exhibit a temperature dependence characteristic of antiferromagnetic exchange coupling. A cluster model for the redox center of Chromatium HiPIP that appears compatible with the PMR and preliminary x-ray results$^4$-$^{11}$ is discussed.

Among the iron–sulfur proteins that can be isolated from the photosynthetic bacterium Chromatium$^1$ is an electron-transfer agent with an unusually high, positive redox potential ($E_m = +0.35$ V)$^2$ known as HiPIP (for high-potential iron protein). HiPIP has a molecular weight of 10,074, and contains 4 atoms of iron and "labile" sulfide and 4 cysteine residues per mole protein. This protein has been the subject of a number of physical studies, including x-ray analysis,$^4$ esr,$^5$,$^6$ Mössbauer,$^6$ optical, and dichroic$^5$,$^7$ spectroscopy, as well as a magnetic susceptibility determination.$^8$ Mössbauer spectroscopy suggests that the four iron atoms of the protein are equivalent in each of the redox forms.$^5$ The magnetic susceptibility of oxidized HiPIP corresponds to one unpaired electron per molecule of protein, while reduced HiPIP appears diamagnetic.$^9$ The four component iron atoms by x-ray diffraction are in a single unresolved cluster at a resolution of 4 Å.$^4$ We wish to report here pmr (proton magnetic resonance) studies on HiPIP which provide insight into the magnetic and structural characteristics of its component iron–sulfur moiety.

Methods and Materials. HiPIP was prepared from Chromatium Strain D as described by Bartsch.$^1$ The protein was recrystallized twice from 50–55% saturated ammonium sulfate containing 1 mM 2-mercaptoethanol, and desalted. The protein solution was dialyzed exhaustively at 4°C against 99.77% D$_2$O and concentrated by vacuum ultrafiltration. The unbuffered, deuterium-exchanged 4.6 mM solution of reduced HiPIP exhibited an $A_{425}/A_{280}$ of 2.55 at pH 6.6. The HiPIP was oxidized by addition of a 50% molar excess of crystalline K$_4$Fe(CN)$_6$. Excess K$_4$Fe(CN)$_6$ had no
effect on the pmr spectrum of HiPIP and was added only to insure complete oxidation. Pmr spectra were obtained with a Varian 220 MHz spectrometer; the signal-to-noise characteristics of most spectra were improved through the use of a Varian C-1024 computer of average transients. All spectra were internally referenced to the methyl resonances of the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid in units of Hz or parts per million (ppm), with downfield shifts assigned positive values.

**Results.** HiPIP as isolated from *Chromatium* under aerobic conditions in the reduced form does not exhibit electron spin resonance absorption 3,5 and has been reported to be diamagnetic by susceptibility determinations 8. HiPIP may, however, be converted reversibly into a paramagnetic form (S = 1/2) that displays electron spin resonance absorption below 28°K (g_∥ = 2.21, g_⊥ = 2.04) 3,5 upon one-electron oxidation with agents such as ferricyanide.

The pmr spectrum of the oxidized form of HiPIP in solution at 5°C is shown in Fig. 1. Resonance absorption in the -2 to +10 ppm region normally encountered in proteins 9 is observed in the pmr spectrum of oxidized HiPIP. In addition, five temperature-dependent resonances in the 20-45 ppm region of resonance absorption are observed in the 5°C spectrum. Characteristics of these five resonances at 5°C and 40°C are shown in Fig. 2, and their temperature dependences are plotted in the left-hand portion of Fig. 3.

Contact-shifted resonances have been observed in the extreme low-field region of resonance absorption of both oxidized and reduced forms of ferredoxin from

![Fig. 1. 220 MHz pmr spectrum of oxidized Chromatium HiPIP at +5°C. Spectrum referenced with respect to internal 2,2-dimethyl-2-silapentanesulfonic acid. The strong peak at 5.0 ppm arises from residual HDO protons. The inset is a 100-pass, computer-averaged spectrum of the 22-43 ppm region of resonance absorption.](image)

![Fig. 2. Contact-shifted resonances of oxidized Chromatium HiPIP at 5 and 40°C. The 5°C spectrum was accumulated on a computer of average transients for 100 passes, the 40°C spectrum for 150 passes.](image)
Clostridium pasteurianum that have been assigned to the \( \beta \)-CH\(_2\) protons of the eight component cysteine residues.\(^9\) The five resonances of Fig. 2 observed in the pmr spectrum of oxidized HiPIP similarly are assigned a contact interaction origin on the basis of their extreme low-field resonance positions and their temperature dependences. The temperature dependences of the assigned contact-shifted resonances are to be compared with the virtual temperature independences of resolved resonances of the aromatic region of resonance absorption of HiPIP (right-hand portion of Fig. 3).

Numbers of protons contributing intensity to the five contact-shifted resonances of HiPIP are indicated in Fig. 3. Each of the four resonances whose contact-shifts decrease with temperature arises from a single proton. These we assign to the \( \beta \)-CH\(_2\) protons of two of the four cysteine residues of HiPIP.\(^6\) The highest and lowest fields of these four resonances tentatively are associated with one cysteine residue, and the other two resonances with a second. As for Clostridium ferredoxin, nonequivalence of \( \beta \)-CH\(_2\) protons of a given cysteine residue, when observed, is attributed to the angular dependence of contact interaction in a rigid system.\(^6\) By this analysis, then, the \( \beta \)-CH\(_2\) protons, of two of the four cysteine residues of oxidized HiPIP are sensing virtually identical spin densities, and observed contact-shifts for these protons exhibit an almost Curie law dependence over the accessible temperature range.

The fifth contact-shifted resonance of oxidized HiPIP arises from three protons and, in contrast to the other four, its shift increases with temperature (Fig. 3). This resonance is assigned to three of the four \( \beta \)-CH\(_2\) protons of the other two cysteine residues of the protein. The (assumed) missing intensity corresponding to one proton is either too broad for detection or has been shifted into an inaccessible region of resonance absorption or into the +3 to +8 ppm region of resonance absorption where identification of a broadened, contact-shifted resonance would be difficult. The breadths of contact-shifted resonances in
HiPIP are quite variable and can exceed 250 Hz (see Fig. 2). For this reason we tentatively attribute to strong electron–nucleus dipolar interaction our inability to detect the postulated missing intensity.

Discussion. The striking feature of the pmr spectrum of oxidized HiPIP is that four of the resolved contact-shifted resonances exhibit near Curie law behavior while the magnitude of the fifth contact-shifted resonance increases with temperature. On the basis of the above analysis, the four cysteine residues of oxidized HiPIP are divided into two classes, each containing two residues. One class exhibits Curie law behavior that is compatible with the measured magnetic susceptibility. Contact shifts, however, of the two cysteine residues of the other class increase with temperature, a characteristic which in the case of *Clostridium* ferrodoxin has been attributed to antiferromagnetic exchange interaction between iron atoms.

A model for the Fe/S/cysteine moiety of HiPIP that appears compatible with the x-ray structural results to date on the reduced protein is shown in Fig. 4. The four iron atoms and four inorganic sulfur atoms are in a slightly elongated tetrahedral array with the iron atoms at the vertices of the tetrahedron and the inorganic sulfur atoms above the faces. Each of the four cysteine residues of the polypeptide chain is coordinately bound to an iron atom via sulfur. Antiferromagnetic exchange interaction is presumed to occur by way of direct Fe–Fe interaction and/or superexchange through the four coordinately bound inorganic sulfur atoms. An organometallic analog to this structure would appear to be Fe₄S₄(C₅H₆)₄, whose structure has been determined. In this analog, a cyclopentadienyl group rather than sulfur of cysteine is bound to each of the iron atoms.

Before proceeding to a rationalization on the basis of the structure of Fig. 4 of the previously presented pmr results on oxidized HiPIP, we will discuss the results of pmr studies on reduced HiPIP. The low-field portion of the pmr spectrum of reduced HiPIP at +5 and +40°C is shown in Fig. 5, and the temperature dependences of the chemical shifts of these resonances as well as those of the higher field aromatic protons are plotted in Fig. 6. No resonances were observed for reduced HiPIP between 20 and 45 ppm and between −2 and −33 ppm. The temperature dependences of the aromatic protons (1400–2000 Hz) of reduced HiPIP as plotted in Fig. 6 are typical of those noted for protons...
exhibiting resonance absorption in the −2 to +8 ppm range for both redox forms of HiPIP.

By susceptibility determination, reduced HiPIP to 200°K in frozen solution is diamagnetic. Yet the three lowest field resonances of reduced HiPIP, each resonance corresponding in intensity to a single proton, clearly from resonance position and temperature dependence owe their origins to contact-shift interaction. This in turn implies the presence of at least some degree of paramagnetism. In the absence of conformational changes, temperature dependences of contact shifts usually parallel that of the magnetic susceptibility. The increase of contact shifts of reduced HiPIP with temperature implies a thermally accessible state or states more paramagnetic than the ground state. All pmr observations on reduced HiPIP are compatible with antiferromagnetic coupling of the iron atoms in the ground state with, however, the exchange interaction being such that a nonzero population of a magnetic state is possible over the 5–80°C temperature range. Over the temperatures examined in the susceptibility determination, the population of a magnetic state could have been so low as to make detection of a paramagnetic contribution impossible.

Strong antiferromagnetic coupling in reduced HiPIP would appear to be compatible with the cluster structure of Fig. 4. However, as previously discussed, upon one-electron oxidation to oxidized HiPIP with $S = \frac{1}{2}$, two of the four cysteine residues exhibit contact shifts compatible with antiferromagnetic exchange interaction and two display Curie law contact-shift temperature dependences. If our pmr assignments are correct and if, indeed, Fig. 4 does approximate the structure of the redox center of HiPIP, an explanation of the pmr spectrum of oxidized HiPIP would be that the electron is removed from one end of the redox center. The single unpaired electron could be considered as primarily if not totally delocalized over only two iron atoms, two inorganic sulfurs, and two cysteine sulfurs. The $\beta$-CH$_3$ protons of these latter two cysteine residues would be those that exhibit approximate Curie law behavior. Temperature dependences of contact shifts of the $\beta$-CH$_3$ protons of the other two cysteine residues would be expected to be dominated by the underlying antiferromagnetic exchange coupling of the redox cluster. This exchange coupling might well be different in the reduced and oxidized forms of HiPIP, as is sug-

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**Fig. 6. Temperature dependences of the low-field resonances of reduced HiPIP. Numbers of protons associated with certain of these resonances are indicated. The dotted lines represent resonances that disappear with time in D$_2$O solution and presumably, therefore, arise from exchangeable protons.**
gested from comparison of Figs. 6 and 3, because of differences in electronic structure and/or geometry.

The above analysis implies that for a given HiPIP molecule in the oxidized form, the unpaired electron or "hole" remains localized at one end of the redox cluster for a time exceeding $10^{-4}$ sec. Otherwise, a uniform temperature dependence to the contact shifts for the $\beta$-CH$_3$ protons of all four cysteine residues would be expected, rather than the two distinctly different dependences observed. This conclusion, however, appears to be in conflict with Mössbauer results which indicate all four iron atoms to be equivalent in the oxidized form. Assuming that the pmr analysis is correct, the Mössbauer and pmr results could still be compatible if removal of an electron from the iron–sulfur cluster were not a sufficiently strong perturbant to discriminate by isomer shift and quadrupole coupling constant the two nonequivalent pairs of iron atoms in oxidized HiPIP. Such could conceivably be the case if the electron removed from reduced HiPIP upon oxidation were from an orbital centered primarily on the sulfur rather than iron atoms.

Abbreviations: HiPIP, high potential iron protein; pmr, proton magnetic resonance.

* Contribution no. 1704.
† Association of contact-shifted resonances observed for HiPIP and Clostridial ferredoxin with the $\beta$-CH$_3$ protons of cysteine residues, while reasonable, remains to be unequivocally established. This we hope to accomplish by examination of the pmr spectrum of at least one iron–sulfur protein in which the $\beta$-CH$_3$ protons are replaced with deuterium.