Hydrogen-1 Nuclear Magnetic Resonance of the Nitrogenase Iron Protein (Cp2) from *Clostridium pasteurianum*

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ABSTRACT: Proton NMR spectra (250 MHz) of the nitrogenase iron protein from *Clostridium pasteurianum* (Cp2) were found to display 9 or 10 paramagnetically shifted resonances in the 15–50 ppm range. The most shifted resonances belonged to two approximately equal subsets having temperature dependences of opposite sign. The latter occurrence is consistent with the interaction of the corresponding protons with an antiferromagnetically coupled metal center. The number of proton resonances of Cp2, their positions, and their temperature dependences were similar to those observed in spectra of $(4Fe\text{-}4S)^+$ ferredoxins, particularly those of the latter that contain a single tetranuclear cluster, such as the ferredoxin from *Bacillus stearothermophilus*. The effects of several adenine nucleotides on the paramagnetically shifted proton resonances of Cp2 have been investigated. Whereas MgAMP had no effect at all, MgADP and MgATP were found to induce different modifications, which in both cases involved approximately half only of the shifted proton resonances. These data suggest that nucleotide binding affects mainly one part of the iron–sulfur cluster. A remarkable feature of the spectra of Cp2 in the presence of MgATP is the grouping of the shifted proton resonances in sets of two or four having identical chemical shifts and temperature dependences. A nearly perfect 2-fold symmetry is thus suggested for the arrangement of the cysteine protons around the active site. These observations lend support to the proposal that the (4Fe-4S) cluster is held symmetrically between the two identical subunits and are consistent with the existence of two MgATP binding sites on nitrogenase iron proteins. The paramagnetically shifted proton resonances of Cp2 were independent of solvent composition and, in particular, of conditions that changed the relative concentrations of the $S = {}^1/_2$ and $S = {}^3/_2$ spin states displayed by the iron–sulfur cluster at temperatures below 30 K. In addition, the bulk magnetic susceptibility of Cp2 solutions was found to remain largely constant over a wide range of solvent compositions. These observations, together with the similarity of the shifted proton NMR spectra of Cp2 and of *B. stearothermophilus* ferredoxin, strongly suggest that native, as well as nucleotide-bound, nitrogenase iron proteins assume at room temperature a spin state similar to that of the $(4Fe\text{-}4S)^+$ proteins displaying only a $S = {}^1/_2$ ground spin state at low temperature. The proton NMR and EPR results described here are discussed in light of previously published data on synthetic analogues of protein active sites. It is therefrom inferred that the spin-state mixture observed in nitrogenase iron proteins at low temperature is mainly a consequence of the freezing process.

The reduced tetranuclear iron–sulfur clusters $(4Fe\text{-}4S)^+$ display in most cases a $S = {}^1/_2$ ground spin state in proteins (Cammack et al., 1977) as well as in synthetic analogues (Lane et al., 1977). However, the occurrence of higher multiplicity half-integer spin states has been demonstrated in some synthetic clusters (Laskowski et al., 1979; Carney et al., 1986, 1988) and in a few proteins (Zimmerman et al., 1978; Vollmer et al., 1983; Moulis et al., 1984; Lindahl et al., 1985, 1987). These high spin states were discovered and characterized by a combination of EPR,[1] Mössbauer, and, in some cases (Lindahl et al., 1985, 1987; Carney et al., 1986, 1988), magnetic susceptibility measurements, all of which were carried out on frozen solutions or in the solid state. Therefore, nothing is known about the significance of the $S > {}^1/_2$ spin multiplets in liquid solution at room temperature.

Among the $(4Fe\text{-}4S)^{2+/+}$ proteins with high spin states, a particularly interesting one is the iron protein of nitrogenase, which functions as a specific reductant of the molybdenum–iron (MoFe) protein in the overall reduction of dinitrogen to ammonia (Orme-Johnson, 1985). A number of apparently conflicting data on the active site of the reduced iron protein from *Azotobacter vinelandii* (Av2) [reviewed in Lindahl et al. (1985)] have recently been clarified by the demonstration that it consists of a $(4Fe\text{-}4S)^+$ cluster with either a $S = {}^1/_2$ or a $S = {}^3/_2$ ground spin state at low temperature, the two spin states being interconvertible by changing the solvent composition (Lindahl et al., 1985, 1987). The biochemical significance of this spin-state mixture has so far remained undetermined since there is no apparent correlation between spin state and enzymatic activity (Lindahl et al., 1985) or redox potential (Morgan et al., 1986). No evidence has yet been obtained for the occurrence at room temperature of the spin-system heterogeneity observed at low temperature.

Valuable information on the latter point may be expected from ^1H NMR spectroscopy, which is particularly suited to the investigation of paramagnetic systems in liquid aqueous solutions since it can use the protons neighboring metal centers as probes of their magnetic state. ^1H NMR spectra of (4Fe-4S) proteins (Phillips & Poe, 1973; Packer et al., 1977;

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[1] Abbreviations: Cp, *Clostridium pasteurianum*; Cp2, nitrogenase iron protein from *C. pasteurianum*; Av2, nitrogenase iron protein from *Azotobacter vinelandii*; CD, circular dichroism; EPR, electron paramagnetic resonance; Fd, ferredoxin; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; TSTP, sodium 3-(trimethylsilyl)-tetradeuteriopropionate.

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Nagayama et al., 1983; Markley et al., 1986) display numerous magnetically shifted resonances attributed to C_α and C_β protons of cysteine residues coordinated to the cluster. We have recently used ^{1}H NMR spectroscopy to probe the paramagnetic centers of reduced 2(4Fe-4Se)$^+$ clostridial ferredoxins at room temperature (Gaillard et al., 1987). These spectra exhibit features arising from two different spin-state ladders (Gaillard et al., 1987) reminiscent of the coexistence of distinct spin states at low temperature (Moulis et al., 1984; Gaillard et al., 1986; Auric et al., 1987).

A similar set of data on nitrogenase iron proteins would be critical in evaluating a possible physiological role of the $S = {}^3/_2$ spin state. We have therefore collected ^{1}H NMR spectra of the reduced nitrogenase iron protein from *Clostridium pasteurianum* (Cp2) in various solvents that are known (Lindahl et al., 1985) to strongly modify the relative ratios of the $S = {}^1/_2$ and $S = {}^3/_2$ spin states at low temperature. We have also measured the bulk magnetic susceptibility of the protein in the same conditions. The present NMR results suggest that the active site of Cp2 displays at room temperature a spin-state ladder similar to that occurring in proteins for which only a $S = {}^1/_2$ ground state is populated at low temperature, as evidenced by EPR and Mössbauer spectroscopy. ^{1}H NMR spectra of Cp2 have also been recorded in the presence of MgADP and MgATP, and they indicate that the proton environment of the active site senses specifically the binding of both nucleotides.

Materials and Methods

The growth of *C. pasteurianum* W5 (ATCC 6013) cells, the purification of the nitrogenase components, and the measurement of their activity were as previously described (Meyer, 1981). The specific activity of the Fe protein (Cp2) was 2000–2200 nmol of C_2H_4 (min·mg)$^{-1}$ in the presence of excess MoFe protein. In such preparations 1 mol of Cp2 released 3 mol of iron in the presence of bathophenanthrolinedisulfonate and MgATP (Ljones & Burris, 1978; Meyer, 1981); i.e., the concentration of (4Fe-4S) active site was 75% that of the protein.

Bacillus stearothermophilus (ATCC 29609) was grown at 55 °C and its ferredoxin isolated as described (Mullinger et al., 1975) except that the preparative polyacrylamide gel electrophoresis was replaced by a precipitation in 90% saturated ammonium sulfate and subsequent chromatography on Ultrogel AcA 202 (LKB) (Gaillard et al., 1986). Samples for NMR measurements were prepared as described below for Cp2.

The preparation and handling of Cp2 samples for NMR measurements were carried out in an anaerobic glovebox (Jacomex, Livry-Gargan, France) in which an oxygen concentration of 1 ppm was maintained.

Solvent exchange was performed by passing 0.3–0.4 mL of Cp2 over a 3-mL column of Sephadex G-25 equilibrated with a deuteriated buffer solution (50 mM potassium phosphate, 100 mM NaCl, 2 mM dithionite) prepared as follows: 78 mg of KH_2PO_4, 340 mg of K_2HPO_4, 300 mg of NaCl, and 18 mg of sodium dithionite were dissolved in 50 mL of D_2O (99.8%, from the Commissariat à l'Energie Atomique, Saclay, France). When dissolved in H_2O, these amounts of phosphate yield solutions having a pH of 7.4, and therefore the pD of the deuteriated solvent was not measured. Subsequent to solvent exchange, appropriate amounts of urea-d_4 (98% isotopic enrichment, Janssen Chimica), of ethylene glycol-d_6 (99% isotopic enrichment, Commissariat à l'Energie Atomique) or of nucleotide solutions in D_2O were added as indicated to Cp2 solutions, and the samples were transferred into 5-mm internal diameter NMR tubes (Wilmad Glass Co.) that were stoppered with rubber septa and carefully sealed with tape. After completion of the NMR measurements (1–2 h), the tubes were returned to the glovebox and aliquots drawn for protein assays, enzymatic activity determinations, and EPR measurements. Protein concentrations were in the 20–40 mg/mL range. The loss of activity was always less than 20%. The EPR spectra of samples taken before and after the NMR spectra were recorded had similar intensities, which indicated that no significant reoxidation of Cp2 took place. The shifted resonances observed in the 15–50 ppm range are significantly broader, at a given temperature, than those occurring in spectra of (4Fe-4S)$^+$ ferredoxins [Figure 1; see also Gaillard et al. (1987) and references cited therein]. Therefore, most of the spectra shown here have been recorded at higher temperatures (in the 42–52 °C range) than usual in order to improve the accuracy of the frequency determinations: The latter point is well illustrated by comparison of the spectra of native Cp2 recorded at 295 and 325 K (Figure 1A,B). As the iron proteins of nitrogenase are insensitive to moderate heating, little if any inactivation was expected in such conditions. We have indeed observed no qualitative nor quantitative alteration of the NMR spectra of Cp2 for periods of 2 h at 52 °C. In addition, the loss of activity was no greater at 52 °C than at 20 °C.

^{1}H NMR measurements were carried out on a Bruker WM-250 spectrometer. Typical spectra resulted from the sum of ca. 40 000 transients, which represented an acquisition time of ca. 40 min. Chemical shifts were referenced to the internal standard sodium 3-(trimethylsilyl)tetradeuteriopropionate (TSTP). Magnetic susceptibilities were measured as described previously (Philips & Poe, 1972; Gaillard et al., 1987).

EPR spectra were obtained with a X-band Varian E-109 spectrometer coupled to a Hewlett-Packard 9826 calculator. An Oxford Instruments ESR 900 flow cryostat was used to adjust the EPR sample temperature (Moulis et al., 1984). The concentration of Cp2 in the EPR samples was in the 20–40 mg/mL range.

Results

^{1}H NMR spectra of reduced Cp2 are shown in Figure 1. Increasing the temperature from 295 to 325 K resulted in a significant narrowing of the lines. Seven paramagnetically shifted proton resonances could be singled out in the 325 K spectrum: three low-field resonances at 46.3, 44.9, and 42.7 ppm, two at 25.6 and 18.1 ppm, a relatively sharp line at 16.5 ppm, and a shoulder at ca. 15.5 ppm on the high-field side of the latter line. No additional resonances were detected low-field of 55 ppm or high-field of 0 ppm. The integrated intensities of the shifted resonances have been measured: If the 18.1 ppm line, which had the lowest intensity, is accounted for by one proton, the 25.6 and 16.5 (including its high-field shoulder) ppm resonances involve two and three protons, respectively, and the three low-field resonances involve three or four protons. Thus, the spectra of Cp2 display altogether 9 or 10 proton resonances in the 15–50 ppm range. The most shifted resonances belong to two approximately equal subsets having temperature dependences of opposite sign (Table I), which is consistent with the interaction of the corresponding protons with an antiferromagnetically coupled metal center (Dunham et al., 1971). The number of proton resonances of Cp2 and their positions are reminiscent of those observed in spectra of (4Fe-4S)$^+$ ferredoxins, particularly those of the latter that contain a single tetranuclear cluster, such as the ferredoxin from *B. stearothermophilus* (Table I, Figure 1C). Furthermore, the signs (Table I) and the absolute values of the temperature dependences of the shifted proton resonances are

Table I: Paramagnetically Shifted ^{1}H NMR Signals of Reduced Cp2[a]

Cp2 (native)	Cp2 (+MgATP)	Cp2 (+Mg-α,β-methylene-ATP)	Cp2 (+MgADP)	*B. stearothermophilus* ferredoxin, reduced
46.9[b] (-)		46.5[b] (-)		46.8 (-)
45.1[b] (-)	42.0 (4) (-)		42.0[b] (-)	43.9 (-)
43.1[b] (-)		43.0[b] (-)	39.1[b] (+)	38.2 (+)
				36.4 (-)
				33.7 (+)
24.9 (2) (+)	25.2 (2) (+)	24.9 (2) (+)	25.6 (2) (+)	20.8 (+)
17.4 (1) (+)	21.7 (2) (+)	20.1 (1) (+)	22.4 (1) (-)	18.0 (+)
16.5 (2) (-)	16.8 (2) (-)	16.7 (2) (-)	16.1 (2) (-)	16.3 (-)
15.5[c]				

[a] Chemical shifts at T = 315 K are given in parts per million relative to the TSTP reference. Unless otherwise specified, the relative positioning, in this table, of the resonances of the various proteins should not be taken as inferring any correlation between their assignments. Magnesium and nucleotide concentrations were 5 mM. Numbers in parentheses indicate the numbers of proton resonances assigned to a given peak. (+) Peaks whose chemical shifts increase with increasing temperature. (-) Peaks whose chemical shifts decrease with increasing temperature. [b] The intensities of these resonances are discussed in the text. [c] Shoulder on the high-field side of the 16.5 ppm resonance.

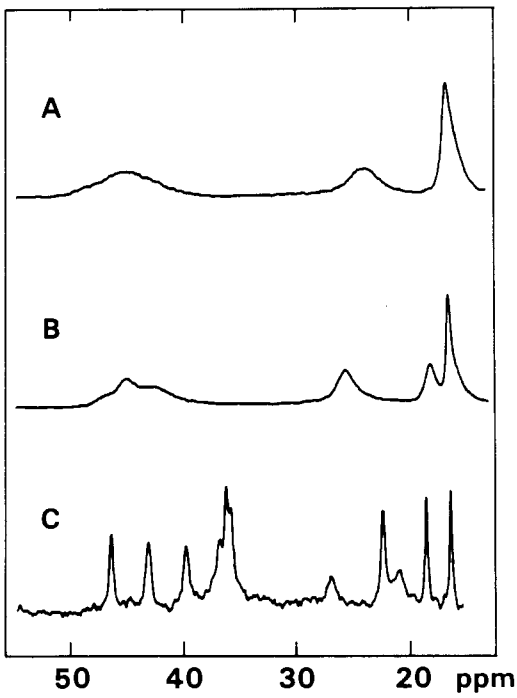

FIGURE 1: Low-field parts of 250-MHz ^{1}H NMR spectra of reduced Cp2 and of reduced *B. stearothermophilus* ferredoxin: (A) native Cp2 at 295 K; (B) native Cp2 at 325 K; (C) *B. stearothermophilus* Fd at 325 K.

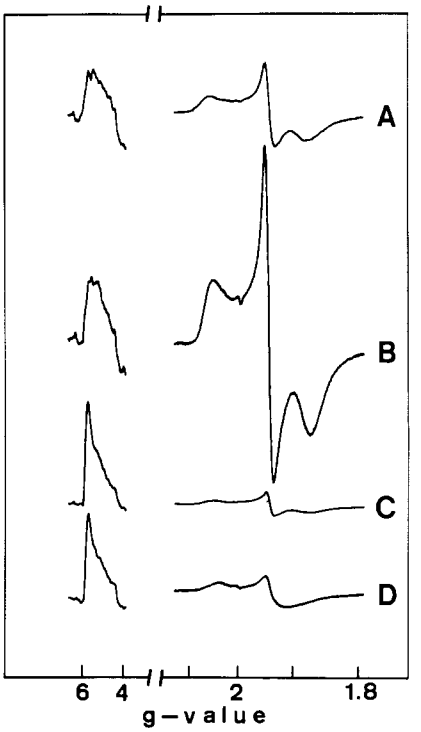

FIGURE 2: Dependence of the EPR spectra of Cp2 on solvent composition: (A) native Cp2; (B) Cp2 in 30% ethylene glycol; (C) Cp2 in 0.5 M urea; (D) Cp2 in 2 M urea. All spectra were recorded with 1.25-mT amplitude modulation, 100-kHz field modulation, and 9.22-GHz klystron frequency. The lower field (g = 4–6) region of the spectra was recorded at 5 K with a microwave power of 5 mW and the higher field ($g \simeq 2$) region at 12 K with a microwave power of 1 mW. The ratio of the gain of the high-field region to that of the low-field region is the same in all spectra.

similar for the two proteins. The main difference between the two spectra concerns the widths of the Cp2 resonances, which are considerably greater than those of the ferredoxin resonances: This, however, is probably arising mostly from increased relaxation broadening in the larger Cp2 protein. Thus, the ^{1}H NMR spectra of Cp2 display the features expected from the interaction of at least 9 or 10 protons with a paramagnetic $(4Fe\text{-}4S)^+$ cluster. A noteworthy feature of these shifted protons is the occurrence of most of them in sets of two to four having very similar chemical shifts (Table I).

Effect of Solvent Composition on NMR Spectra and Bulk Magnetic Susceptibility. It has previously been shown that the mixture of spin states occurring in native Av2 is converted into either nearly pure $S = {}^1/_2$ form in 50% ethylene glycol or nearly pure $S = {}^3/_2$ form in 0.4 M urea (Lindahl et al., 1985). Data consistent with this analysis have also been obtained with the iron proteins from *Azotobacter chroococcum* and *Klebsiella pneumoniae* (Hagen et al., 1985). In analogous conditions, Cp2 behaved in much the same way as the other nitrogenase iron proteins, as witnessed by the EPR spectra of Figure 2, some of which confirm earlier observations by Zumft et al. (1973). We have noted the relative intensity of the $g \simeq 5$ and of the $g \simeq 2$ signals to be highest in 0.2–0.5 M urea and 20–50% ethylene glycol, respectively (Figure 2). Zumft et al. (1973) had also observed that higher concentrations of urea (>1 M) resulted in the redevelopment of the $g \simeq 2$ signal, but with an altered symmetry (axial instead of rhombic).

We have therefore recorded ^{1}H NMR spectra of Cp2 in 30% ethylene glycol (Figure 3B), 0.2 M urea (not shown), 0.5 M urea (Figure 3C), and 2 and 5 M urea (not shown). These spectra did not differ from each other, neither from that of native Cp2 (Figure 3A). The positions and the relative intensities of the shifted proton resonances were identical in all of these spectra. The only noteworthy difference was the poor resolution of the spectrum in 30% ethylene glycol, presumably due to the slower tumbling of protein molecules in the more viscous solvent.

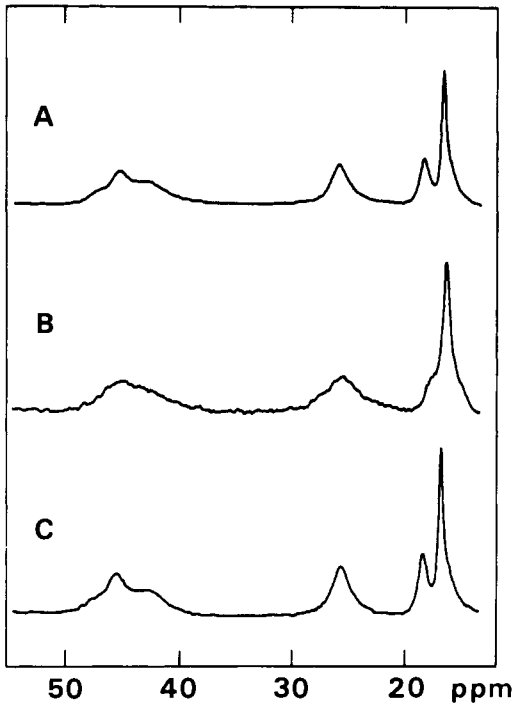

FIGURE 3: Dependence of the low-field parts of ^{1}H NMR spectra of reduced Cp2 on solvent composition: (A) native Cp2; (B) Cp2 in 30% ethylene glycol; (C) Cp2 in 0.5 M urea. All spectra were recorded at 325 K.

Table II: Magnetic Susceptibility of Reduced Cp2: Dependence on Solvent Composition and Nucleotides

	Δf^a (Hz)		Δf^a (Hz)
Cp2 native	9	Cp2 in 0.5 M urea	7
Cp2 + Mg ATP	7	Cp2 in 2 M urea	9
Cp2 + Mg ADP	7	Cp2 in 30% ethylene glycol	10

[a] Change in the proton resonance frequency of the TSTP reference caused by the paramagnetic protein (see Materials and Methods), given for a 1 mM concentration of $(4Fe\text{-}4S)^+$ cluster. Δf arises from contributions of diamagnetic and paramagnetic terms. The uncertainty on the measurement of Δf was mainly due to the errors on the frequency reading (0.3 Hz) and on the protein assay (10–15%) and resulted in a cumulative absolute error of 1.5–2 Hz.

We have also determined the bulk magnetic susceptibility over a wide range of solvent conditions (Table II). The measured magnetic susceptibility of Cp2 mainly consists of a paramagnetic contribution from the iron–sulfur cluster and of a diamagnetic contribution from the polypeptide chain. The latter component is undetermined but may be assumed to remain nearly constant since it is highly unlikely that its variations would exactly cancel out the variations of the paramagnetic component in the various solvent conditions used here. It may therefrom be inferred that the paramagnetic contribution from the $(4Fe\text{-}4S)^+$ cluster is also independent of solvent composition, consistent with the invariance of the chemical shifts and temperature dependences of the paramagnetically shifted resonances (Figure 3).

Effect of Adenine Nucleotides. In the presence of MgADP or MgATP, the paramagnetically shifted proton resonances of reduced Cp2 occurred in the same frequency range as for the native protein (Figure 4). This is consistent with the observation that the solution susceptibility of Cp2 was not modified (Table II) and in addition suggests that no major change of the electron spin density distribution took place upon binding of either nucleotide.

In the presence of MgATP (Figure 4B), the 16.5 and 24.9 ppm resonances were only slightly shifted to lower field, but the 17.4 ppm line disappeared while another resonance having

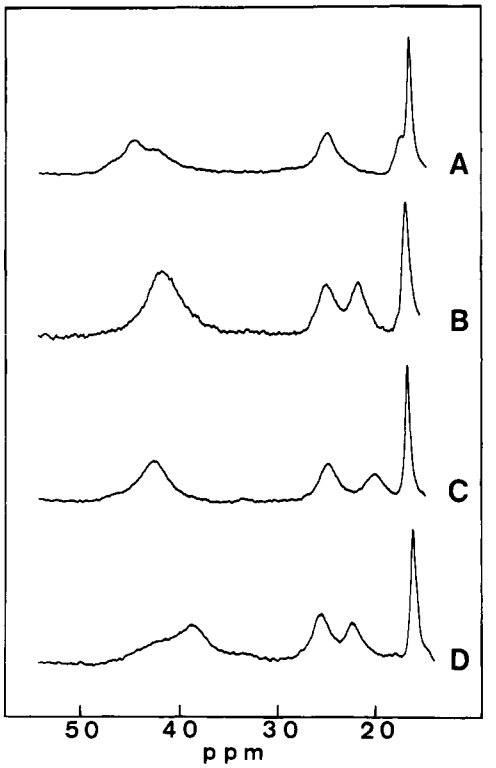

FIGURE 4: 250-MHz ^{1}H NMR spectra of reduced Cp2 in the presence of adenine nucleotides: (A) native Cp2; (B) Cp2 + MgATP; (C) Cp2 + magnesium adenosine α,β-methylenetriphosphate ; (D) Cp2 + MgADP. All spectra were recorded at 315 K. Magnesium and nucleotides were added at concentrations of 5 mM.

the same temperature dependence, but twice more intense, appeared at 21.7 ppm. The lowest field resonances of the native protein were replaced by a broad line at ca. 42 ppm, which had the same temperature dependence as the former ones (Table I, Figure 4). A remarkable feature of the spectra in the presence of MgATP was the grouping of the shifted proton resonances in only four peaks having intensity ratios of 2:1:1:1 from low field to high field. One has therefore to consider the possibility that only five paramagnetically shifted proton resonances might occur in the presence of MgATP. However, by recording spectra of the same sample of Cp2 before and after the addition of MgATP and by comparing their intensities, we have found that the same numbers of shifted protons were involved in both cases (within the uncertainty of the measurement, 10–15%). It is therefrom inferred that 10 paramagnetically shifted resonances are present in spectra of Cp2 + MgATP, with a remarkable grouping in pairs having identical chemical shifts and temperature dependences (Figure 4B, Table I).

Upon addition of MgADP (Figure 4D), the chemical shifts of some resonances changed in a somewhat similar way as with MgATP. However, the 16.5 ppm resonance was slightly shifted to higher field (e.g., in the opposite direction as with MgATP), and the temperature dependences of some resonances (at 22.4 and 39.1 ppm) were opposite to those of the resonances occurring in the same frequency range in spectra of native Cp2 or Cp2 + MgATP (Table I). Thus, the binding of MgADP resulted in a more pronounced perturbation than suggested by the mere positions of the resonances. The integrated intensity of the paramagnetically shifted resonances of Cp2 in the presence of MgADP amounted to the same number of protons (9 or 10) as in the case of native Cp2. Unlike in the case of MgATP, the shifted protons in the spectra of Cp2 + MgADP were not all associated in pairs.

The specificity of the changes induced by MgATP and MgADP was assessed by the observation that MgAMP caused no modification of the NMR spectra of Cp2. Furthermore, upon addition of apyrase (EC 3.6.1.5), which hydrolyzes both ATP and ADP into AMP, to solutions of Cp2 + MgATP or Cp2 + MgADP, the corresponding spectra did revert to that of native Cp2.

We have also investigated the effects of two structural analogues of ATP: In the presence of magnesium adenosine β,γ-methylenetriphosphate, the NMR spectra of reduced Cp2 were identical with those observed in the presence of MgATP (not shown). This analogue had previously been shown to modify the EPR spectra (Zumft et al., 1973) and the redox potential (Watt et al., 1986) of nitrogenase iron proteins in a similar way as did MgATP and to inhibit the turnover of nitrogenase (Weston et al., 1983). Another analogue, magnesium adenosine α,β-methylenetriphosphate, had previously been found to be without effect on EPR spectra of Cp2 (Zumft et al., 1973). In contrast, its effects on the NMR spectra were noticeable: Some of the lowest field resonances of native Cp2 merged into a single peak at ca. 43 ppm, and the 17.4 ppm line of native Cp2 was shifted to 20.1 ppm (Figure 4C, Table I).

Discussion

The results reported here confirm the high value of ^{1}H NMR as a probe of active-site structure in iron–sulfur proteins. In previous studies on ferredoxins, ^{1}H NMR of paramagnetically shifted proton resonances was mainly used as a means of cluster type identification (Nagayama et al., 1983) and of investigating the polypeptide environment of the active site (Markley et al., 1986) or its magnetic state(s) (Gaillard et al., 1987). In the latter case it proved to be complementary to EPR and Mössbauer spectroscopies in extending the investigated temperature range to physiological conditions.

Information along similar lines has been obtained in the present study of reduced Cp2, the spectra of which display a number of resolved resonances in the 15–50 ppm range. The ^{1}H NMR spectra of Cp2 are sufficiently similar to those of *B. stearothermophilus* Fd (Figure 1, Table I) to confirm that the reduced nitrogenase iron protein contains a $(4Fe\text{-}4S)^+$ cluster (Lindahl et al., 1985) and to suggest that the environment of the latter resembles that of simple ferredoxins. These similarities include the numbers of shifted resonances observed (9 or 10), the field range where they occur (15–50 ppm), their temperature dependences (Table I), and also the presence, among the less shifted resonances, of narrower peaks (the 16.5 ppm line of native Cp2, Figure 1A) that, in the case of some ferredoxins, have been assigned to C_α protons of cysteine ligands (Packer et al., 1977).

The nitrogenase iron protein nevertheless differs considerably from the ferredoxins by its functioning as a specific reductant of the nitrogenase MoFe protein. It is in particular involved in the binding and hydrolysis of MgATP associated with the reduction of dinitrogen to ammonia by the enzyme (Orme-Johnson, 1985). Indeed, the Fe protein is known to undergo conformational changes upon binding of MgATP or MgADP (Orme-Johnson, 1985). MgATP lowers the redox potential (Zumft et al., 1974), increases the reactivity of the cluster iron to chelators (Walker & Mortenson, 1974), alters CD and MCD spectra (Stephens et al., 1979), decreases the ^{57}Fe quadrupole splittings in Mössbauer spectra (Lindahl et al., 1985), converts the $g = 1.94$ EPR signal from axial to rhombic symmetry (Orme-Johnson et al., 1972; Smith et al., 1973; Zumft et al., 1973), and destabilizes crystals of the protein (Rees & Howard, 1983). MgADP has similar effects as MgATP (Zumft et al., 1973, 1974; Lindahl et al., 1985), but it neither increases the sensitivity of the cluster to chelators (Walker & Mortenson, 1974) nor destabilizes crystals of the protein (Rees & Howard, 1983). Our present ^{1}H NMR data are consistent with these data inasmuch as MgATP and MgADP induce different modifications of the paramagnetically shifted proton resonances (Figure 4, Table I). The observation that some resonances remain largely unmodified in the presence of either MgATP or MgADP (Figure 4, Table I) would suggest that nucleotide binding affects mainly one part of the cluster. A remarkable feature of the spectra of Cp2 in the presence of MgATP is the grouping of the shifted proton resonances in sets of two or four having identical (within the resolution of the spectra) chemical shifts and temperature dependences (Figure 4B). A nearly perfect 2-fold symmetry is thus suggested for the arrangement of the cysteine protons around the active site, as well as for the distribution of the electron spin density. These observations lend support to the proposal that the (4Fe-4S) cluster is held symmetrically between the two identical subunits (Hausinger & Howard, 1983) and are consistent with the existence of two MgATP binding sites on nitrogenase iron proteins (Orme-Johnson, 1985). The presence of lines accounting for only one proton per protein in the NMR spectra of native Cp2 and Cp2 + MgADP suggests that the active site assumes a somewhat lower symmetry than in the presence of MgATP. However, the observation that several protons surrounding the iron–sulfur cluster are nearly insensitive to the presence of nucleotides indicates that the binding of the latter probably does not induce drastic structural changes in the immediate vicinity of the active site.

The $(4Fe\text{-}4S)^+$ cluster of reduced *B. stearothermophilus* Fd is known to assume the classical $S = {}^1/_2$ spin state at low temperature (Mullinger et al., 1975), in contrast to several nitrogenase iron proteins that display a mixture of $S = {}^1/_2$ and $S = {}^3/_2$ spin states (Lindahl et al., 1985; Hagen et al., 1985; Watt & McDonald, 1985). However, at room temperature, the numbers and positions of the paramagnetically shifted proton resonances of *B. stearothermophilus* Fd and of Cp2, as well as their temperature dependences, are similar (Figure 1, Table I). Furthermore, all of the above-mentioned parameters and also the magnetic susceptibility (Table II) remain unchanged when Cp2 solutions contain either 30% ethylene glycol or 0.5 M urea, which increase the relative concentrations of the $S = {}^1/_2$ or of the $S = {}^3/_2$ spin states, respectively, in frozen solution (Figure 2). This set of data strongly suggests that in Cp2, and most probably in the other nitrogenase iron proteins, the paramagnetic center responsible for the magnetic properties at room temperature assumes a spin-state ladder similar to those occurring in the proteins that display a single $S = {}^1/_2$ ground spin state at low temperature. In the presence of nucleotides, the frequency range of the paramagnetically shifted proton resonances, the magnitudes of their temperature dependences, and the bulk magnetic susceptibility do not differ significantly from those of the native protein. It may therefrom be inferred that the different forms of nucleotide-bound iron protein evidenced at low temperature (Lindahl et al., 1987) merge into a single species, the active site of which displays at room temperature a spin-state ladder originating from a classical $S = {}^1/_2$ ground state. Thus, the same situation is encountered here as with native Cp2.

A quite different situation has been evidenced in the case of reduced Se-substituted clostridial ferredoxins (Moulis & Meyer, 1982), where the spin-state heterogeneity observed below 30 K (Moulis et al., 1984; Gaillard et al., 1986; Auric et al., 1987) is reflected in the room temperature ^{1}H NMR

spectra by the occurrence of distinct spin-state ladders (Gaillard et al., 1987). The relative intensities of the EPR signals of the various spin states are sensitive to variations in solvent composition in the case of nitrogenase iron proteins (Lindahl et al., 1985) but insensitive in the case of the Se-substituted clostridial ferredoxins (Gaillard et al., 1986). Thus, the presence of several spin systems appears to be an intrinsic property of $2(4Fe\text{-}4Se)^+$ ferredoxins in frozen as well as in liquid solutions, whereas it rather seems to arise from interactions between the protein and the frozen solvent in the case of the nitrogenase iron protein.

The role of the freezing process in the occurrence of two spin states in reduced Cp2 is most probably important, if not exclusive, in view of the following observations. The EPR spectra of some $(2Fe\text{-}2X)^+$ (X = S, Se) synthetic clusters ($S = {}^1/_2$ ground spin state) were found to display variations in the main values of their g-tensors, depending on the solvents in which they were frozen (Beardwood & Gibson, 1983). Concerning the tetranuclear clusters, which are more relevant here, it has recently been shown that the $(4Fe\text{-}4X)^+$ (X = S, Se) synthetic compounds assuming a $S = {}^1/_2$ and those having a $S = {}^3/_2$ ground spin state display only minor differences in tridimensional structure (Carney et al., 1988): Thus, spin-state conversions in this type of cluster do not seem to require larger structural changes than those that may occur at the protein active site in the frozen solvent. Indeed, we have observed (unpublished results) that the $S = {}^1/_2$:$S = {}^3/_2$ ratio in Cp2 can be modified, with all solvent compositions described in Figure 2, by equilibrating the EPR samples at ca. 230 K (acetonitrile–dry ice) after freezing them in liquid nitrogen and putting them back at 77 K without thawing. For instance, with 0.5 M urea as solvent, the procedure described above results in a 2.5-fold increase of the $S = {}^1/_2$ signal intensity and a 1.4-fold decrease of the $S = {}^3/_2$ signal intensity. These data strongly suggest that the spin-state mixture in Cp2 results from interactions between the $(4Fe\text{-}4S)^+$ active site and its protein and solvent environment in the frozen state. According to our NMR results, these interactions are far weaker or nonexistent in liquid solution. Among biological systems, the possibility of detecting more than one spin species for a given active-site geometry is not restricted to the nitrogenase iron proteins: In the case of the S_2 state of the oxygen-evolving complex of photosystem II (Zimmerman & Rutherford, 1986; Brudvig, 1987), the EPR signals attributed to $S = {}^3/_2$ and $S = {}^1/_2$ spin states can be generated upon illumination of the S_1 state. The $S = {}^3/_2$ signal has been found to be converted into the $S = {}^1/_2$ multiline signal when the temperature was increased.

The present work and the examples mentioned above suggest that the spectroscopic data obtained at cryogenic temperatures should when possible be cross-checked with room temperature data in order to derive structural characteristics in functional conditions.

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Registry No. MgADP, 7384-99-8; MgATP, 1476-84-2; nitrogenase, 9013-04-1; L-cysteine, 52-90-4.

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Function of Dopachrome Oxidoreductase and Metal Ions in Dopachrome Conversion in the Eumelanin Pathway†

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ABSTRACT: The conversion of dopachrome (DC) in the eumelanin pathway has been analyzed to determine the specific product and the role of enzyme control. 5,6-Dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) were quantitated by HPLC with fluorescent detection, after DC incubation with heated and unheated preparations of B-16 melanoma derived dopachrome oxidoreductase (DCOR). The enzyme-catalyzed reaction produced DHICA as the major product, while DHI formed with the spontaneous reaction. It had originally been suggested that the major product of DC conversion was DHI, with DHICA being formed as a minor product of this conversion [Raper, H. S. (1927) *Biochem. J. 21*, 89-96]. Copper, nickel, and cobalt ions promoted conversion of DC, with nickel simulating DCOR activity. Removal of free ions from unheated DCOR did not alter DC conversion. We conclude that the major product of DC conversion is DHICA and that DCOR is responsible for this conversion.

Eumelanin production is known to proceed through a reaction pathway, the initial steps of which are catalyzed by the enzyme tyrosinase (Raper, 1928). Although the steps beyond the action of tyrosinase were originally believed to occur spontaneously, recent studies have shown that the conversion of DC[1] is under enzymatic control (Korner & Pawelek, 1980; Barber et al., 1984). The enzyme responsible for this conversion, DCOR, was extracted from purified melanosomes and shown to be separable from tyrosinase by enzyme activity and gel filtration (Barber et al., 1984). Initial characterization of the enzyme showed it to be protease-sensitive and heat-labile, with an optimum pH range of pH 6-8. The molecular mass of DCOR was estimated by AcA22 gel filtration to be 34 000 daltons. Further characterization awaits purification of the enzyme. The original assay for DCOR activity used light spectroscopy to measure the decrease in absorbance as DC (red color) was converted to a colorless compound, but this method was not capable of detecting the specific reaction products. According to the classical Raper–Mason pathway, the major product of DC conversion is DHI (Mason, 1955), with DHICA being formed as a minor product of this conversion (Raper, 1927); however, several recent studies have indicated that this concept of the eumelanin pathway is not correct and that DHICA may be the major product of DC conversion.

When [^{14}C]carboxy-labeled dopachrome was used as substate for DCOR, Korner and Pawelek observed that the loss of DC color did not directly correlate with $^{14}CO_2$ liberation, suggesting that DC was converted to DHICA rather than DHI (Korner & Pawelek, 1980). Korner and Gettins (1985) then analyzed the stable colorless product of DC conversion using nuclear magnetic reasonance and mass spectroscopy and identified the compound as DHICA. Finally, Ito has recently reexamined the structure of eumelanin polymer and has found that it contains a high quantity of carboxy-containing indoles. To further investigate the products of the enzymatic conversion of DC, we have developed an assay using HPLC with fluorescent detection. In this paper, the results of using the assay to quantitate the enzymatic and nonenzymatic formation of DHICA and DHI, as well as the effect of metal ions on DC conversion, are described.

Materials and Methods

Preparation of DCOR from Melanosomes. (A) Maintenance of B-16 Melanoma. Melanotic B-16 melanoma lines were maintained by serial transplantation in C57BL/6J mice. A suspension of approximately 10^5 tumor cells in minimal essential media was injected in subcutaneous tissue of the lower

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[1] Abbreviations: DC, dopachrome; DCOR, dopachrome oxidoreductase; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; HPLC, high-performance liquid chromatography.

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