Proton magnetic resonance studies of 7Fe ferredoxins

Three redox states of the [4Fe–4S]cluster in a *Pseudomonas ovalis* ferredoxin

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The oxidizability of a redox couple, [4Fe–4S], in a 7Fe ferredoxin extracted from *Pseudomonas ovalis* was monitored by ^{1}H-NMR. The iron-sulfur cluster in the ferredoxin was not only reducible (Nagayama et al., 1983) but also oxidizable in its native form. This result provided the first verification of 3 redox states for a redox center in ferredoxin, 4Fe, in the native form of the protein.

NMR *Ferredoxin* *[4Fe–4S]* *Redox state* Pseudomonas ovalis

1. INTRODUCTION

The three-state theory [1] has long been a guiding principle for interpretation of the redox states of iron-sulfur clusters in ferredoxins. A decade ago many efforts were made to obtain direct evidence supporting the theory in intact proteins. With the high-potential protein (HiPIP) type of ferredoxin (Fd) this was partly successful by monitoring the ESR signal associated with oxidation and reduction [2]. One flaw in that experiment, however, was that the reduction was carried out in denatured protein with an 80% DMSO/20% H_2O binary solvent.

Recent NMR studies with 7Fe ferredoxins revealed that *Pseudomonas ovalis* Fd, a homologous protein of the well-known *Azotobacter vinelandii* Fd I, was reducible in its 4Fe redox center [3]. A 4Fe center in *A.vinelandii* Fd was already known to show a spectroscopic change associated with oxidant titration [4]. To attribute the latter phenomenon to oxidation of the redox center, we must first rule out possibilities other than the oxidation reaction. For example, the structural conversion from 4Fe to 3Fe as observed in a ferredoxin from *Bacillus stearothermophilus* [5,6] must be dismissed. Such a core conversion was actually observed in the 7Fe ferredoxins from *P.ovalis, Mycobacterium smegmatis* and *Thermus thermophilus* when excess ferricyanide was added [7]. With a stoichiometric amount of ferricyanide, however, *P.ovalis* Fd showed a behavior different from the *M.smegmatis* and *T.thermophilus* ferredoxins [7]. Here, details of the study on the oxidizability of the 4Fe center are reported. Together with the results of reduction experiments [3], we confirmed that the 4Fe redox center in *P.ovalis* Fd could take 3 states in its native form. To our knowledge, this is the first verification of the three-state theory of an iron-sulfur redox center in the native form of a protein.

2. MATERIALS AND METHODS

P.ovalis Fd was purified as in [8]. Ferricyanide titration was carried out for a ferredoxin sample of 0.5 mM by adding a small amount of 50 mM potas-

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sium ferricyanide solution at room temperature. Solvent exchange to D_2O buffer and concentration of the sample solutions were done using an Amicon ultrafiltration device with a YM5 membrane under a nitrogen pressure of 3–4 atm. A deuterated 1/15 M phosphate buffer was prepared from sodium pyrophosphate and KH_2PO_4 solutions adjusted to pH* 8.6. Here, pH* indicates the uncorrected pH meter reading.

After ferricyanide was removed from the solution with the ultrafiltration device, the ferricyanide titrated sample was reduced by adding reductants such as dithionite and sodium borohydride (with methyl viologen) or leaving the sample in air at 4°C for about 1 week.

^{1}H-MNR spectra were obtained using a Bruker 360 MHz (WM-360 wb) spectrometer. Chemical shifts were referenced to 2,2,4,4-tetradeutero-3-(trimethylsilyl)propanesulfonic acid. Free induction decays were added to a total of 4000–40 000 with a repetition cycle of 0.35 s. A frequency resolution of 4.41 Hz was employed.

3. RESULTS

The resonances appearing in the spectral region downfield of 10 ppm provided good probes to monitor the potentiometric change of the iron-sulfur cores [3,6,7]. As shown in fig.1a, 6 contact shifted resonances appeared in this region for *P.ovalis* Fd. The results of oxidation and reduction experiments are shown in fig.1b–f. With addition of ferricyanide equimolar to the ferredoxin (fig.1b), the peak profile changed as A3, A4, A5 and A6 peaks decreased in their intensities and new peaks O1 and O2 appeared. Ferricyanide in an about 2-fold molar ratio to the ferredoxin completed the change and the A3–A6 peaks were completely replaced by the O1 and O2 peaks (fig.1c). From the temperature dependence of the chemical shift, peak O1 was attributed to the same proton as the A3 peak, which was around the 3Fe center [3,7]. On the other hand, peak O2 was considered to be a new resonance representing protons around the oxidized 4Fe center because it showed a temperature dependence completely different from those of the peaks which had disappeared (A4–A6).

The results of the reduction experiments are shown in fig.1d–f. Fig.1d demonstrates the peak profile returning to the isolated state with dithio-

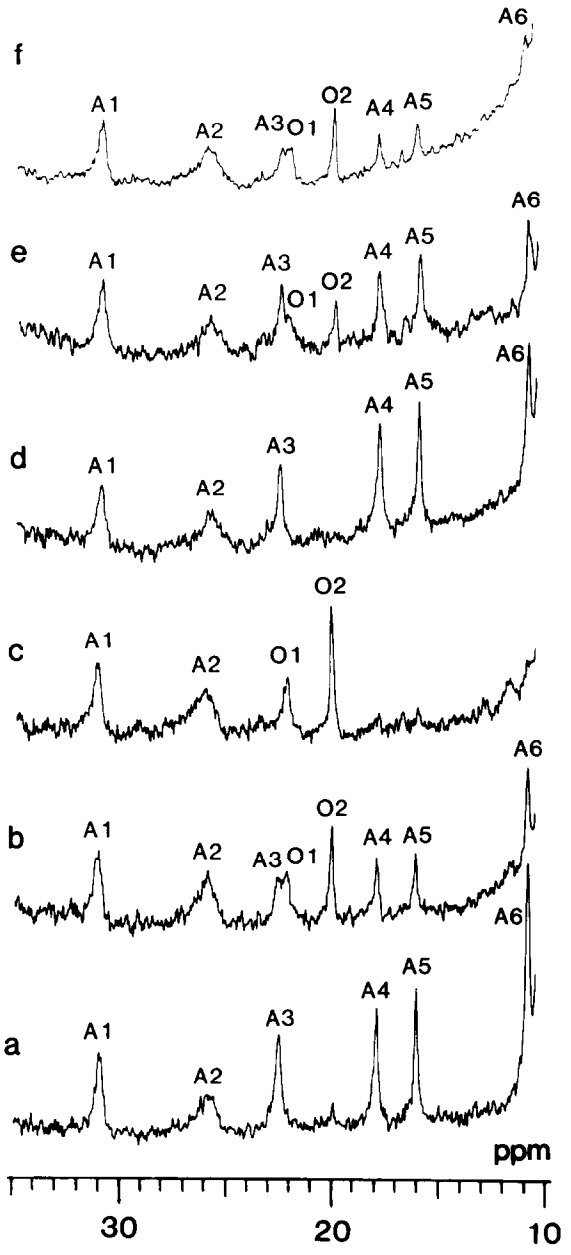

Fig.1. Downfield shifted resonances of *P.ovalis* ferredoxin recorded with 360 MHz NMR. *T*, 27°C; pH* 8.6: (a) 0.6 mM Fd as isolated, 10^3 scans; (b) 0.6 mM Fd + 0.5 mM ferricyanide, 10^3 scans; (c) 0.6 mM Fd + 1.1 mM ferricyanide, 10^3 scans; (d) 0.3 mM oxidized Fd + 0.55 mM dithionite, 4×10^3 scans; (e) 0.3 mM oxidized Fd + solid borohydride (with methylviologen), 4×10^3 scans; (f) 0.2 mM oxidized Fd after air equilibration at 4°C for about 1 week, 10^4 scans.

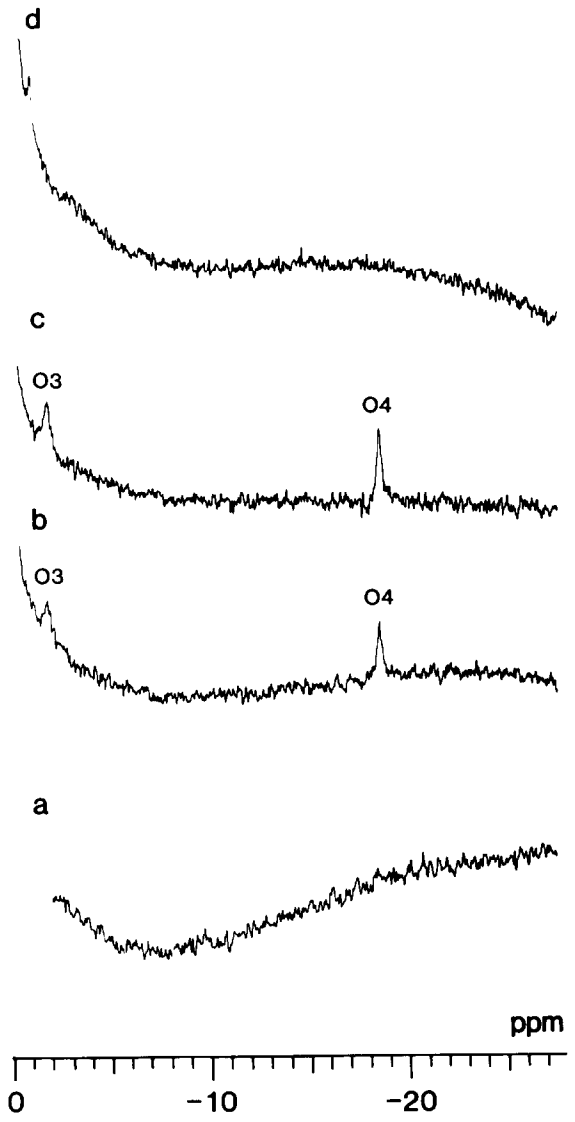

Fig.2. Upfield shifted resonances of *P.ovalis* Fd recorded with 360 MHz NMR. *T*, 27°C; pH* 8.6. (a–d) conditions are the same as described in fig.1, with corresponding alphabetical order.

nite in an about 2-fold molar ratio to the ferredoxin. Adding a minute amount of solid borohydride together with the redox mediator methyl viologen resulted in the spectrum of fig.1e. Almost equal peak heights of the two peak groups (O1,O2) and (A4–A6), indicated that the solution was midway in its return to the isolated state. Equilibration of the oxidized ferredoxin with air might bring the sample back to the reduced state as isolated, if strong oxidants were removed in the sample solution. This experiment is shown in fig.1f. After long-term incubation of the sample in air, ferredoxin as isolated reappeared without adding any specific reductants.

As pointed out in [9], the upfield contact shifted resonances may give a unique clue to identifying the oxidized state of the 4Fe core. In the upfield region two candidates for the contact shifted resonances were found as shown in fig.2. In the 0–50 ppm region no resonances were observed for *P.ovalis* Fd as isolated (fig.2a). Two resonances designated O3 and O4 appeared in that region on adding a stoichiometric amount of ferricyanide (fig.1b,c). These peaks disappeared again on addition of dithionite (fig.1d). The chemical shifts of the hyperfine shifted resonances which characterize each stage of the NMR spectra are summarized in table 1.

Table 1

Chemical shifts of contact-shifted resonances of *P.ovalis* Fd in the isolated and oxidized states (*T* 27°C, pH* 8.6)

As isolated			Oxidized	
4Fe	4Fe or 3Fe	3Fe	4Fe	3Fe
17.7, 10.5 (A4) (A6)	15.9 (A5)	30.9, 25.7, 22.3[a] (A1) (A2) (A3)	19.8, −2.0, −18.9 (O2) (O3) (O4)	30.9, 25.7, 21.9[a] (A1) (A2) (O1)

[a] Temperature dependence of the chemical shifts and conservation of the total areas of O1 and A3 peaks suggests that both peaks originate from the same proton. Designation of peaks is shown in parentheses.

4. DISCUSSION

In [7] a reversible spectroscopic change in the 4Fe center prior to the irreversible core conversion was reported. To establish whether the change arose from the real oxidation process of the redox center, 3 tests were made: (i) stoichiometry of reversible oxidation–reduction reaction; (ii) reduction of the oxidized ferredoxin with various reductants such as dithionite, borohydride and air equilibration; and (iii) search for the upfield contact shifted resonances which characterize the oxidized state of ferredoxins [9]. Judging from the results of these 3 tests, *P.ovalis* Fd can be safely claimed to be oxidizable. This, together with the result in [3], provided the first experimental confirmation of Carter's three-state theory for the intact (native) form of iron-sulfur proteins. In other words, we verified that the true basis for the classification of the ferrodoxins was not simply their redox potentials. The 4Fe center in *P.ovalis* and *A.vinelandii* Fd was once classified as HiPIP type. But such a classification is illogical because the redox center has two redox couples, high and low. Just as in other ordinary proteins, homology in the primary to tertiary structures seems to be a more important basis for the classification of iron-sulfur proteins. In this context, *P.ovalis, A.vinelandii, T.thermophilus* and *M.Smegmatis* ferredoxins are claimed to make one type of 7Fe ferredoxin. They probably have common features in physical properties of redox reactions. Reversible oxidation might also be feasible in the 4Fe centers of *T.Thermophilus* and *M.smegmatis* ferredoxins, if appropriate oxidation conditions are found.

Two peaks, A3 and O1, were considered to originate from the same proton around the 3Fe center but to have different chemical shifts in the two redox states. The two peaks were clearly separated in the NMR spectrum at the stage when the two redox components were mixed (fig.1b). The frequency difference of the two peaks indicates that the interconversion speed between the two redox states is much slower than $10^3\ s^{-1}$.

The peak assignment of A5 to one of the two iron centers is still inconclusive. We have contradicted earlier results that the peak disappears in association with the reduction of 3Fe center [3], the core conversion from 3Fe to 4Fe center [7] and now the oxidation of 4Fe center. A proton close to both centers, whether or not ligated to the redox center, may explain such an apparent contradiction. We observed a resonance having properties similar to the A5 peak in two other 7Fe ferredoxins [3,7].

In conclusion, two redox reactions with high and low potentials were finally found for a 4Fe center in the iron-sulfur protein in its native conformation.

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