Proton magnetic resonance studies of 7Fe ferredoxins

Conversion of a 4Fe core to a 3Fe core with ferricyanide

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The redox couples in 7Fe ferredoxins (Fd) treated with ferricyanide were monitored by ^{1}H-NMR. An excess amount of ferricyanide was found to effect conversion of one of the two redox centers, the 4Fe core, to a 3Fe core in the ferredoxins extracted from *Thermus thermophilus, Mycobacterium smegmatis* and *Pseudomonas ovalis*. On long term incubation in air, the converted 3Fe core showed even further change in NMR spectra.

NMR *Ferredoxin* *3Fe core* *4Fe core* *Iron-sulfur*

1. INTRODUCTION

Recent NMR studies with 7Fe ferredoxins extracted from 3 bacterial species revealed that two redox centers, the 4Fe and 3Fe cores, in the protein were both reversibly reducible [1]. This result has cast doubt on the widely accepted concept that 7Fe ferredoxins include a high-potential type of 4Fe core. To date, change in a 7Fe ferredoxin, *Azotobacter vinelandii* Fd I [2], monitored with visible absorption and ESR spectra on addition of ferricyanide has been interpreted as unique evidence of the redox couple. To reexamine whether such a spectroscopic change arises either from the oxidation process or from an irreversible chemical change at the redox center concerned, the NMR method was applied and spectra were recorded of the 3 7Fe ferredoxins from *Thermus thermophilus* [3], *Mycobacterium smegmatis* [4] and *Pseudomonas ovalis* [5]. An irreversible change, the core-conversion from 4Fe to 3Fe, has been reported for *Bacillus stearothermophilus* Fd [6,7]. An NMR study with *A. vinelandii* Fd I also reported an irreversible process occurring in the solution treated with ferricyanide [8].

Contact-shifted resonances in the low-field region were monitored to follow reactions associated with ferricyanide addition. Except for the case of the stoichiometric oxidant added to the *P. ovalis* Fd solution, excess ferricyanide always induced irreversible change in the samples at the site of the 4Fe cluster. Based on the core classification with NMR spectra [7], conversion from 4Fe to 3Fe was concluded for the 3 ferredoxins. (For the 3Fe core a novel type of structure, [3Fe-4S], was recently proposed from the chemical analyses of iron and sulfide, and EXAFS experiments of aconitase [9]. This new iron-sulfur cluster was also claimed to be common in ferredoxins containing the 3Fe center such as *A. vinelandii, T. thermophilus* and *Desulfovibrio gigas* Fds with

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resonance Raman spectroscopy [10]. As yet, NMR cannot provide a decisive method to distinguish between the two possibilities for the 3Fe core, say [3Fe-3S] or [3Fe-4S], and so simply the term 3Fe core is used to refer to the iron-sulfur clusters.) In the long-term aerobic incubation of ferricyanide-treated samples, another conversion reflected in the spectral change was observed.

2. MATERIALS AND METHODS

T. thermophilus Fd [3], *M. smegmatis* Fd [11] and *P. ovalis* Fd [12] were purified as described. Ferricyanide treatment was carried out for stock solutions of ~0.5 mM with $K_3Fe[CN]_6$ to about 10 molar excess to ferredoxin. Immediately after the addition of ferricyanide, incubation was performed at 0°C for 5 h. Solutions were adjusted to pH 8.0 for *M. smegmatis* Fd and *P. ovalis* Fd and to pH 8.5 for *T. thermophilus* Fd. *T. thermophilus* Fd was very stable and highly resistant to the oxidant under various pH and temperature conditions [13]. To induce an oxidant reaction in the *T. thermophilus* Fd sample, dithionite reduction must take place just before adding excess solid ferricyanide. Solutions for NMR measurements were adjusted to pH* 8.6 for *P. ovalis* Fd and *M. smegmatis* Fd and pH* 9.0 for *T. thermophilus* Fd in deuterated 1/15 M phosphate buffer prepared from $Na_4P_2O_7$ and KH_2PO_4 solutions. pH* indicates the uncorrected pH meter reading. Solvent exchange to D_2O buffer, ferricyanide desalting and the concentration of sample solutions were done using an Amicon ultrafiltration device with a YM5 membrane under a nitrogen pressure of 3–4 atm. Final concentration of the solutions was roughly estimated as below 1 mM.

Ferredoxins in NMR tubes (5ϕ) were reduced with solid $Na_2S_2O_4$ under nitrogen flow. Special care was taken to seal NMR tubes under anaerobic conditions. Successively after the reduction, reoxidation of the samples with air was performed in situ by desealing and stirring the NMR tubes.

Proton NMR spectra were obtained using a Bruker 360 MHz (WM-360 wb) spectrometer. Chemical shifts were referred to 2,2,4,4-tetradeutero-3-trimethylsilylpropanesulfonic acid. Free induction decay (FID) signals were added to total 4000–40000 scans with a repetition cycle of 0.35 s. A resolution of 4.41 Hz was employed to record the spectra.

3. RESULTS

NMR spectra of less than 10 ppm for the 3 samples are shown in fig.1. The resonances appearing in the illustrated region provided good probes to monitor core structures and potentiometric change of the cores [1,2,7]. The bottom traces (i-a, ii-a, iii-a) in the figure show spectra of the intact form of 7Fe ferredoxins as isolated; the 6 contact-shifted resonances, peaks A1–A6, were resolved. After ferricyanide treatment the spectra of the samples changed as shown in traces, i-b, ii-b and iii-b. Resonances of A1–A3 remained unchanged but peaks A4–A6 were replaced by new peaks designated as B1–B4 or B5. Through the NMR measurements under different conditions, assignment of the same numbers to distinct peaks in separate experiments was determined by the chemical shifts of peak positions and the temperature dependence of the shifts. The unchanged peaks A1–A3 were assigned to the protons near the 3Fe core existing in the isolated form of ferredoxins [1]. Some reaction, therefore, should occur at the 4Fe site. To characterize the reaction, reduction-reoxidation experiments followed. Traces from i-c to iii-c show the complete reduction of ferricyanide-treated ferredoxins by dithionite. Disappearance of multiple peaks was observed through the 3 samples. This suggested the first change induced by the oxidant as arising from some irreversible change at the 4Fe site, very probably core-conversion from a 4Fe cluster to a 3Fe cluster, rather than an oxidation process [7].

The spectra monitored in the course of reoxidation were slightly more complicated. In the short-term process of reoxidation with air which was performed at room temperature (25°C) for 5–10 h, besides A (A1–A3) and B peaks, a set of new peaks designated D1–D4 (peak D4 of *T. thermophilus* Fd was also observed under other experimental conditions) appeared in the 3 samples (i-d to iii-d). On long-term incubation at 4°C for about 2 weeks, the B peaks disappeared (i-e to iii-e) indicating lower stability of the B component as compared with the third component represented by the D peaks. In the reoxidation of *P. ovalis* Fd, peaks

A4–A6 which are characteristic of the 4Fe core also reappeared indicating a reconversion. Comparison of two spectra of III-d and III-e suggested greater stability of the intact 4Fe core than the converted 3Fe core in *P. ovalis* Fd. Destructive side reactions were observed in all oxidation-reduction experiments. By tracing the change in intensity of peaks A1–A3, we found that *T. thermophilus* Fd was the most resistant to degradation and that *P. ovalis* Fd was rapidly destroyed.

4. DISCUSSION

4.1. *Core conversion and 6Fe ferredoxins*

Oxidation-reduction experiments verified the irreversible ferricyanide attack upon the 4Fe core. Structureless spectra observed on reduction indicate an irreversible process in ferricyanide treatment. Completely and partially reduced 7Fe ferredoxins in their intact forms are known to show contact-shifted peaks of less than 10 ppm [1]. Judging from the chemical shifts and peak inten-

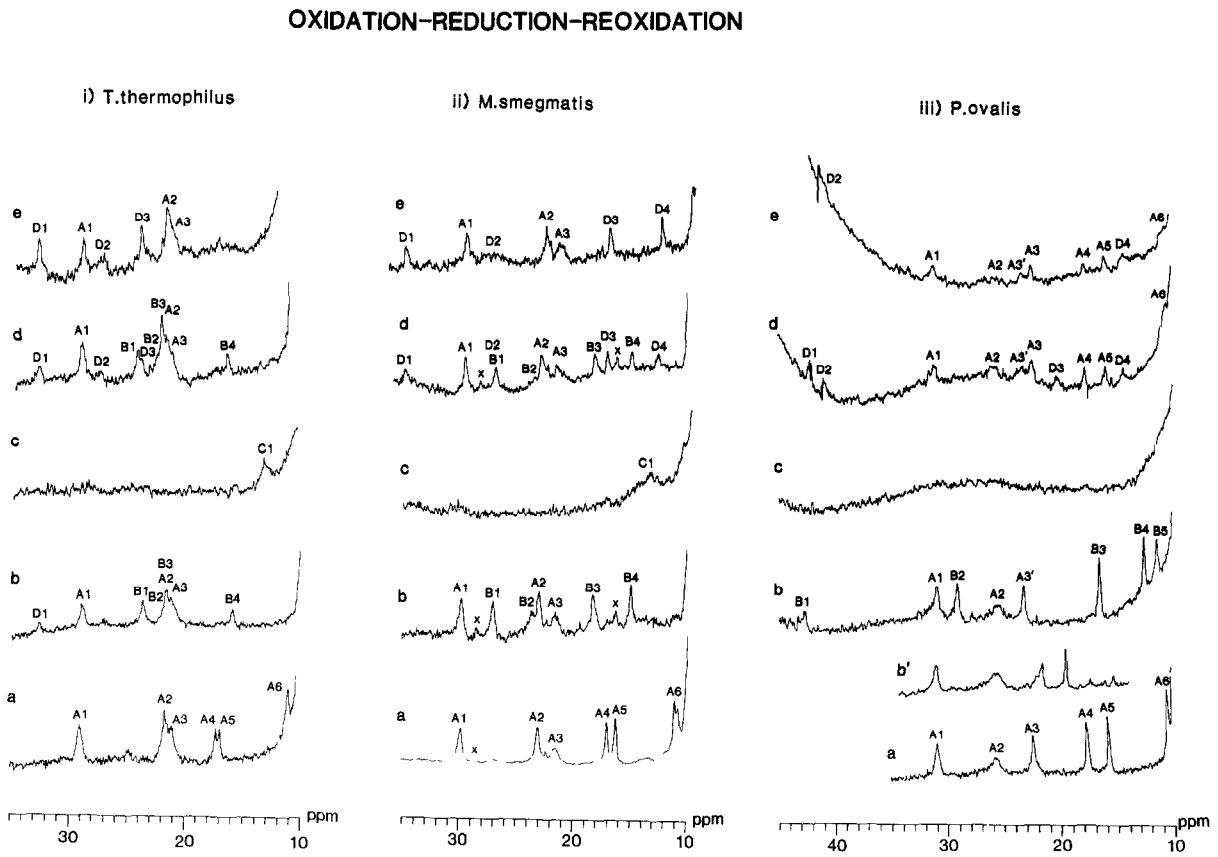

Fig.1. (i) Low-field region of 360 MHz ^{1}H NMR of *T. thermophilus* Fd. pH* 9.0, 27°C. Number of scans: (a) 4×10^3, (b) 4×10^3, (c) 4×10^3, (d) 4×10^3, (e) 4×10^3. (ii) Low-field region of 360 MHz ^{1}H NMR of *M. smegmatis* Fd. pH* 8.6, 27°C. Number of scans: (a) 4×10^3, (b) 10^4, (c) 10^4, (d) 10^4, (e) 10^4. (iii) Low-field region of 360 MHz ^{1}H NMR of *P. ovalis* Fd. pH* 8.6, 27°C. Number of scans: (a) 10^4, (b') 4×10^3, (b) 10^4, (c) 10^4, (d) 4×10^4, (e) 4×10^4. (a) As isolated, (b') oxidized with a stoichiometric amount of ferricyanide in a separate experiment, (b) ferricyanide treated, (c) reduced with dithionite of the ferricyanide treated, (d) reoxidized with air from the reduced at 25°C for 5–10 h, (e) reoxidized from the reduced at 4°C for 2 weeks. From the pH and temperature dependence, peak A3' in *P. ovalis* Fd was found to be a peak shifted from peak A3. x indicates minor peaks with which we are not concerned here.

sities of the two sets of resonances, A and B peaks, and the disappearance of the contact shift in the reduced state (spectra I-c to III-c), the ferricyanide-treated ferredoxins are believed to comprise a new 6Fe ferredoxin which includes two 3Fe cores in a single polypeptide. By the gradual addition of dithionite, the individual potentials of the two 3Fe redox centers were distinguished.

In the course of 4Fe to 3Fe conversion, peak A5 completely lost its intensity. This led us to regard it as representing a proton around the 4Fe site. On the other hand, peak A5 disappeared on reduction of the intact Fds, where another iron core site, 3Fe, was first reduced, suggesting the resonance from a proton around the 3Fe redox center [1]. These two results made it difficult to determine to which site of the iron core, 3Fe or 4Fe, the A5 peak should be attributed. The apparent contradiction could be resolved by taking into account the proximity of the two iron-sulfur clusters in a protein. Namely, if we place emphasis on the result of the reduction experiment, peak A5 should be assigned to a proton around the original 3Fe and the disappearance of A5 in ferricyanide treatment could be explained by a high-field shift of the peak introduced with magnetic interaction through local structural arrangement associated with the core-conversion. Let us bear in mind that some local deformation of the polypeptide structure is inevitable in the core-conversion because rearrangement of ligands is mandatory to hook the 3Fe core in a polypeptide. By switching the roles of the two redox centers, another assignment is also feasible. In this case the influence of the 3Fe site reduction on a proximal ligand of the 4Fe site should be strongly considered. No conclusion has yet been drawn as to which of these possibilities is most likely.

4.2. *Two distinct spectra of the converted 3Fe cluster*

Another interesting feature observed in the reduction-reoxidation experiment is the appearance of a set of new peaks, D peaks. For the sake of convenience we denote Fd α (A peaks) for the 7Fe ferredoxin, Fd β (B peaks) for the 6Fe ferredoxin after ferricyanide treatment and Fd δ (D peaks) for the new ferredoxin after long-term reoxidation. Data are not shown here but the contact shifts of *T. thermophilus* Fd δ and *M. smegmatis* Fd δ disappeared on dithionite reduction. Therefore D peaks are believed to arise from an iron-sulfur core of the 3Fe type.

Here we note the higher stability of Fd δ than that of Fd β and the order of two conversions starting from the intact ferredoxins, Fd α. The species Fd β can be regarded as an intermediate or a transient species of the final stable product Fd δ. In this study we cannot assign a unique answer to the 3 possibilities for the interpretation of the intermediate state; (1) an intermediate of the core structure, (2) an intermediate step of the ligand reshuffling and (3) a redox intermediate. If we take the first, a new structure, [3Fe-4S], proposed in [9,16] could be postulated as an intermediate in the course of the conversion from [4Fe-4S] to [3Fe-3S]. If we accept the second, two ligand arrangements proposed for the [3Fe-4S] structure [9] might become candidates for the two spectroscopically distinguishable states. Distinct but fairly common spectral features observed in the reduction-oxidation experiments (fig.1b,d,e) suggest the commonality of the core structure between the two states. As proton NMR can positively monitor the conformation of the polypeptide part, the ligand rearrangement can give a good explanation of the spectral change despite the conserved core structure. We also cannot deny the third possibility that Fd β is a redox intermediate though very improbable because of its high stability after 5–10 h incubation with air. In table 1 the chemical shifts of low-field resonances at the various redox stages are summarized for the 3 ferredoxins. Fairly good correlation is seen in chemical shift not only between the two types of 3Fe cores in Fds β and δ but also among the intact and converted 3Fe cores in Fds α, β and δ.

4.3. *Oxidizability of* P. ovalis *Fd*

With excess potassium ferricyanide, an irreversible change in the 4Fe cluster was observed. The reversible oxidation process was, however, reported for a 7Fe ferredoxin from *A. vinelandii* Fd I [2,8]. To investigate the oxidizability of 7Fe Fds, especially *P. ovalis* Fd which is the most homologous to *A. vinelandii* Fd I, we applied ferricyanide oxidation with a stoichiometric amount of the oxidant. Though detailed results are not given here, the significant result that the ferredoxin can be reversibly titrated with ferricyanide and dithionite (or borohydride) should be mentioned.

Table 1

Chemical shifts of contact-shifted resonances of 3 7Fe ferredoxins before and after ferricyanide treatment

Ferredoxins	As isolated (Fd α)	Ferricyanide treated[a] (Fd β)	Reduced (Fd β)	Reoxidized[b] (Fdβ + Fd δ) (*T. thermophilus, M. smegmatis*) (Fd α + Fd δ) (*P. ovalis*)
T. thermophilus	29.0, 21.8, 21.1, 17.4 17.0, 11.1	29.1, 23.9, 22.1, 21.7 21.7, 21.1, 16.2	13.0	32.7, 29.0, 27.2, 23.7 23.9, 22.1, 21.8, 21.8 21.1, 16.2
M. smegmatis	29.8, 22.9, 21.5, 17.1 16.2, 11.0	29.7, 27.1, 23.8, 22.8 21.5, 18.1, 15.1	13.5	34.9, 29.8, 27.5, 27.1 23.8, 22.8, 21.6, 18.2 17.1, 15.1, 12.6
P. ovalis	30.9, 25.7, 22.3, 17.7 15.7, 10.5	42.7, 30.9, 29.0, 25.5 23.2, 16.6, 12.5, 11.4	not observed	41.8, 40.7, 30.9, 25.6 23.1, 22.2, 20.0, 17.6 15.7, 14.2, 10.8

[a] From the temperature dependence and pH titration the chemical shifts underlined were identified as arising from the protons around the converted 3Fe core (B peaks)

[b] Chemical shifts underlined correspond to those of D peaks

T = 27°C; pH* = 9.0 (*T. thermophilus*), 8.6 (*M. smegmatis* and *P. ovalis*)

The spectrum for the ferricyanide-titrated state of *P. ovalis* Fd is illustrated in trace b′ of fig.1 (iii). Disappearance of peaks A4–A6 and the appearance of two peaks between A3 and A4 characterized the state of *P. ovalis* Fd. The very naive interpretation of the results mentioned must be oxidation of the Fd in its 4Fe site which has been claimed [2,8]. However the spectral change illustrated here is inconsistent with the NMR result obtained for the oxidized high-potential Fd, *Chromatium vinosum* HiPIP [17]. More investigation is needed to confirm the oxidizability of the 4Fe iron-sulfur cluster in 7Fe Fds.

In conclusion, we propose that the 7Fe ferredoxins are convertible by ferricyanide to 6Fe ferredoxins including two 3Fe cores, one of which is changed from the 4Fe cluster. In the reduction-reoxidation process the converted 3Fe core takes one more state distinctive in NMR spectra. Two forms of 3Fe clusters, [3Fe-4S] (intermediate) and [3Fe-3S] (final), or two ligand arrangements in the [3Fe-4S] structure are thought to correspond to the two 3Fe states.

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