Proton magnetic resonance studies of 7 Fe ferredoxins

Lower potential of the [4 Fe–4 S] redox center than the [3 Fe–3 S] cluster

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Two types of iron–sulfur clusters, [3 Fe–3 S] and [4 Fe–4 S], were identified by ^{1}H-NMR in ferredoxins from *Thermus thermophilus, Mycobacterium smegmatis* and *Pseudomonas ovalis*. The [4 Fe–4 S] clusters always showed the redox couples which had potentials lower than that of the [3 Fe–3 S] clusters.

NMR *Ferredoxin* *Potential* *[3 Fe–3 S]* *[4 Fe–4 S]*

1. INTRODUCTION

Ferredoxins (Fds) extracted from *Thermus thermophilus* [1], *Mycobacterium smegmatis* [2] and *Pseudomonas ovalis* [3] have amino acid sequences homologous to that of *Azotobacter vinelandii* Fd [4]. *M. smegmatis, P. ovalis* and *A. vinelandii* Fds were once classified by iron and sulfur analysis as 8 Fe ferredoxins similar to a small ferredoxin from *Peptococcus aerogenes* [1,2,5] and *T. thermophilus* Fd was postulated to include 2 Fe and 4 Fe iron centers from an EPR study [6]. After the Mössbauer [7] and X-ray [8] studies recently done for *A. vinelandii* Fd, such classifications were widely questioned. Nowadays, 4 homologous ferredoxins are believed to comprise a new class which have one [3 Fe–3 S] and one [4 Fe–4 S] cluster within single a polypeptide. A characteristic EPR signal of $g = 2.01$ observed at the isolated state is a key to identifying the [3 Fe–3 S] cluster [9]. A recent NMR study [10] using various types of ferredoxins proposed another approach to the spectroscopic identification of the clusters. Based on these results and an NMR study on *A. vinelandii* Fd [11], the 3 Fe and 4 Fe cores were sought in the ferredoxins, *T. thermophilus, M. smegmatis* and *P. ovalis* Fd.

Another topic dealt with in this note is the re-examination of a high redox potential (+320 mV) attributed to the [4 Fe–4 S] cluster in a 7 Fe ferredoxin, *A. vinelandii* Fd [12]. For the other two 7 Fe ferredoxins, *T. thermophilus* [5] and *M. smegmatis* [13] Fds, two redox couples were reported in the low potential region below 0 mV. To resolve this contradiction, Carter's 3-state theory [14] was applied, though care must be taken in the interpretation of experimental results with ferricyanide because it often induces degradation or conversion in target samples. The finding of 4 Fe–3 Fe core conversion in *Bacillus stearothermophilus* Fd [15] has provided an example. Before accepting the interpretation with the 3-state theory, we experimentally asked whether very low redox potential arising from the [4 Fe–4 S] cluster exists or not. We succeeded in completely reducing all of the 7 Fe ferredoxins (from *T. thermophilus, M. smegmatis* and *P. ovalis*) using one of the strongest reductants, sodium borohydride.

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2. MATERIALS AND METHODS

Thermus thermophilus [1], *Mycobacterium smegmatis* [13] and *Pseudomonas ovalis* [16] Fds were purified as described. Solutions were adjusted at pH* 8.6 for *P. ovalis* and *M. smegmatis* Fds and pH* 9.0 for *T. thermophilus* Fd in a deuterated 1/15 M phosphate buffer prepared from a sodium pyrophosphate ($Na_4P_2O_7$) solution and a potassium phosphate (KH_2PO_4) solution. The symbol pH* indicates the uncorrected reading of a pH meter. Solvent exchange to D_2O buffer and the concentration of sample solutions was 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 (5) were reduced with solid dithionite ($Na_2S_2O_4$) or a mixture of solid dithionite and solid sodium borohydride ($NaBH_4$) under argon flux. Special care was taken to seal NMR tubes in an anaerobic condition.

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-(trimethylsilyl) propanesulfonic acid. FIDs were added to total 2000–6000 scans with a repetition cycle of 0.35 s. A spectral resolution of 4.41 Hz was employed to record the spectra.

3. RESULTS

NMR spectra downfield of 10 ppm for the 3 samples are shown in fig.1. The resonances appearing in the region arise from the labile amide protons and/or the protons around Fe–S cores. In the ferredoxins of this group appearance of slowly exchanging protons in this region is very seldom. One exception is a minor peak appearing at the right side of peak 6 for *M. smegmatis* Fd (ii: a,b and d). Therefore we can utilize those resonances as monitors to trace the core structures and their redox reactions. From a comparison of the NMR spectra of different ferredoxin, certain character-

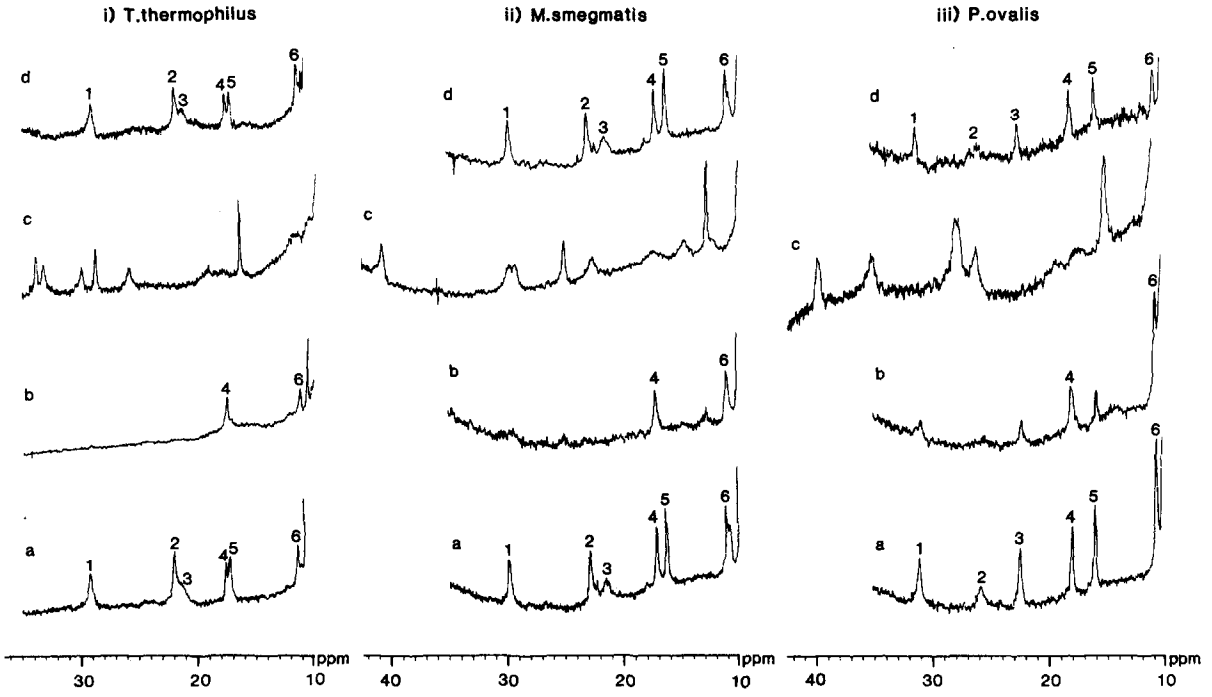


Fig.1. (i) Low-field region of 360 MHz ^{1}H-NMR of *T. thermophilus* Fd. pH* 9.0, 27°C. (ii) Low-field region of 360 MHz ^{1}H-NMR of *M. smegmatis* Fd. pH* 8.6, 27°C. (iii) Low-field region of 360 MHz ^{1}H-NMR of *P. ovalis* Fd. pH* 8.6, 27°C (a,b,d), 17°C (c). (a) Isolated; (b) partially reduced with dithionite; (c) completely reduced with dithionite and sodium borohydride; (d) reoxidized with air following completely reduction.

istics common through the three samples are recognized. First, they involve 3 redox states (a–c). At the isolated state (a) there are 6 contact-shifted resonances designated from 1–6. At the middle reduction state (b) two resonance lines, 4 and 6, remain from the 6 lines. At the final reduced state (c) many lines newly appear with a wide range of shifts. The second characteristic is the reversible reoxidization from the completely reduced state (d). In the course of reduction and reoxidization slight sample degradation was observed. *T. thermophilus* Fd was the most resistant and *P. ovalis* Fd was the most vulnerable.

4. DISCUSSION

4.1. *Cluster identification and potential assignment*

The recent classification study of ferredoxins [10] presented a way to identify the type of Fe–S clusters by investigating the change of NMR characteristics through reduction. This method simultaneously accomplishes the core identification and potential assignment. The change of NMR spectra observed during the first reduction is that of the [3 Fe–3 S] cluster. Several contact-shifted resonances (1, 2, 3 and 5 in fig.1) appear in the region as low as 30 ppm at the isolated state and disappear when reduced. Recent NMR work on *A. vinelandii* Fd [11] assumed the 3 Fe core responsible for the redox couple of −420 mV [12]. The NMR characteristics of the 4 Fe core are quite different from that of the 3 Fe core [10]. A few contact-shifted lines (4 and 6 in fig.1) below 20 ppm appear at the isolated state and instead of them, many new lines come out with a variety of chemical shifts up to 40 ppm when reduced. Therefore the second redox step common to the three ferredoxins could be attributed to arising from the [4 Fe–4 S] cluster. Table 1 summarizes the obtained values of the chemical shifts for the 7 Fe ferredoxins together with the reported chemical shifts for *A. vinelandii* Fd.

In the middle stage of reduction, the spectra showed NMR patterns characteristic to the two successive redox states. For example, minor peaks observed in the partially reduced stage (ii-*b*) indicate that the sample solution includes a small amount of *M. smegmatis* Fd at its completely reduced state. On the other hand, small peaks in the partially reduced *P. ovalis* Fd (iii-*b*) correspond to peaks at the isolated state. Judging from the added amount of dithionite to reduce, the order of three potentials for the redox couple corresponding to the [3 Fe–3 S] cluster was:

$$E^{I}_{m}(P.o.) < E^{I}_{m}(T.t.) < E^{I}_{m}(M.s.) < 0 \text{ mV} \quad (1)$$

Here suffix I denotes the first reduction step. This result is compatible with reported values for the

Table 1

Chemical shifts of contact-shifted resonances of four 7 Fe ferredoxins at 3 redox states (T = 27°C, pH* 8.0–9.0)

Species	3 Fe and 4 Fe cluster oxidized	3 Fe cluster reduced	3 Fe and 4 Fe cluster reduced	Ref.
T. thermophilus	29.0, 21.8, 21.1, 17.4, 17.0, 11.1	17.4, 11.0	33.8, 33.1, 29.8, 28.6, 25.7, 18.8, 16.1	Here
M. smegmatis	29.8, 22.9, 21.5, 17.1, 16.2, 11.0	17.2, 11.0	40.7, 29.8, 29.2, 25.0 22.6, 17.3, 14.7, 12.7	Here
P. ovalis	30.9, 25.7, 22.3, 17.7, 15.7, 10.5	17.8, 10.5	38.6, 36.1, 28.6, 26.8 26.8, 18.0, 18.0, 15.0	Here
A. vinelandii	31.5, 25.8, 22.0, 17.5, 15.7, 11.0[a]	17.5, 16.0 (11.0)[b]	No data available	[11]

[a] Chemical shift of a line recognized in the NMR spectrum of fig.1 [11]

[b] By analogy with the other ferredoxin NMRs, a line with a chemical shift of 11.0 ppm can be observed

redox couples, −250 mV for *T. thermophilus* Fd [6] and −15 mV for *M. smegmatis* Fd [13].

The order of potentials for the redox couple corresponding to the [4 Fe–4 S] cluster was estimated by the added amount of sodium borohydride:

$$E_m^{II}(P.o.) < E_m^{II}(T.t.) < E_m^{II}(M.s.) < -400 \text{ mV} \quad (2)$$

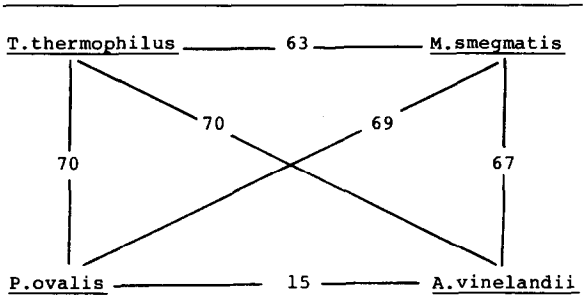
Here suffix II denotes the second reduction step. The order of potentials between *T. thermophilus* and *M. smegmatis* Fds is well explained with the reported values, −530 mV (*T.t.*) [6] and −435 mV (*M.s.*) [13].

4.2. *Is the [4 Fe–4 S] cluster in 7 Fe ferredoxins a cluster of the HiPIP type?*

We are very much concerned with the [4 Fe–4 S] cluster reportedly oxidizable with ferricyanide in *A. vinelandii* Fd [12]. Before directly checking the proposition, we tested whether or not the 4 Fe core has low potential redox couple. (Preliminary NMR experiments to check oxidizability of the [4 Fe–4 S] cluster have been completed and results will soon appear.) NMR has provided evidence that the three Fd samples hold one 4 Fe redox center of low potential type. To extend the result to the case of *A. vinelandii* Fd, sequence homologies among them were compared (table 2). When we compare tables 1 and 2, we can recognize some correlation between the spectral resemblance and the sequence homology. Especially two ferredoxins from *P. ovalis* and *A. vinelandii* quite resemble to each other in sequence and spectrum.

Table 2

Amino acid differences among four 7 Fe ferredoxins [1–4]



106 amino acid length was assumed as a calculation base

We could, therefore, expect the physico-chemical properties fairly common between the two ferredoxins. Estimating from eq. (2) together with the −530 mV potential reported for *T. thermophilus* Fd [6], *P. ovalis* Fd may have a 4 Fe cluster of −600 mV or lower. If such deep potential is the case in *A. vinelandii* Fd, it explains why we have not yet discovered the low potential 4 Fe component in the ferredoxin. Dithionite has been the main reductant utilized to date and is not strong enough to reduce redox centers with potential lower than −600 mV.

We propose that the 7 Fe ferredoxins are a new class of ferredoxin which possess 2 redox centers of low potential type. The potential of the 4 Fe cluster is lower than that of the 3 Fe core. *Azotobacter vinelandii* Fd may not be an exception.

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REFERENCES

[1] Sato, S., Nakazawa, K., Hon-nami, K. and Oshima, T. (1981) Biochim. Biophys. Acta 668, 277–289.
[2] Hase, H., Wakabayashi, S., Matsubara, H., Imai, T., Matsumoto, T. and Tobari, J. (1979) FEBS Lett. 103, 224–228.
[3] Hase, T., Wakabayashi, S., Matsubara, H., Ohmori, D. and Suzuki, K. (1978) FEBS Lett. 91, 315–319.
[4] Howard, J.B., Lorsbach, T.W., Ghosh, D., Melis, K. and Stout, C.D. (1983) J. Biol. Chem. 258, 508–522.
[5] Yoch, D.C. and Arnon, D.I. (1972) J. Biol. Chem. 247, 4514–4520.
[6] Ohnishi, T., Blum, H., Sato, S., Nakazawa, K., Hon-nami, K. and Oshima, T. (1980) J. Biol. Chem. 255, 345–348.
[7] Emptage, M.H., Kent, T.A., Huynh, B.H., Rawlings, T., Orme-Johnson, W.H. and Munck, E. (1980) J. Biol. Chem. 255, 1793–1796.
[8] Stout, C.D., Ghosh, D., Pattabhi, V. and Robbins, A.H. (1980) J. Biol. Chem. 255, 1797–1800.

[9] Johnson, M.K., Spiro, T.G. and Mortenson, L.E. (1982) J. Biol. Chem. 257, 2447–2452.
[10] Nagayama, K., Ozaki, Y., Kyogoku, Y., Hase, T. and Matsubara, H. (1983) J. Biochem., in press.
[11] Sweeney, W.V. (1981) J. Biol. Chem. 256, 12222–12227.
[12] Sweeney, W.V. and Rabinowitz, J.C. (1975) J. Biol. Chem. 250, 7842–7847.
[13] Imai, T., Matsumoto, T., Ohta, S., Ohmori, D., Suzuki, K., Tanaka, J., Tsukioka, M. and Tobari, J. (1983) Biochim. Biophys. Acta 743, 91–97.
[14] Carter, C.W., Kraut, J., Freer, S.T., Alden, R.A., Sieker, L.C., Adman, E. and Jensen, L.H. (1972) Proc. Natl. Acad. Sci. USA 69, 3526–3529.
[15] Bell, S.H., Dickson, D.P.E., Johnson, C.E., Cammack, R., Hall, D.O. and Rao, K.K. (1982) FEBS Lett. 142, 143–146.
[16] Ohmori, D. (1976) Biochem. Biophys. Res. Commun. 72, 566–574.