MÖSSBAUER SPECTROSCOPIC EVIDENCE FOR THE CONVERSION OF [4 Fe–4 S] CLUSTERS IN *BACILLUS STEAROTHERMOPHILUS* FERREDOXIN INTO [3 Fe–3 S] CLUSTERS

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1. Introduction

A large group of iron–sulphur proteins contain [4 Fe–4 S] clusters. These clusters can exist in three possible oxidation levels, normally [4 Fe–4 S]$^{2+,1+}$ in the bacterial ferredoxins (Fds), and [4 Fe–4 S]$^{3+,2+}$ in the high-potential iron proteins (HiPIPS) [1]. It has been proposed [2] that treatment of the 2[4 Fe–4 S] Fd from *Clostridium pasteurianum* with potassium ferricyanide produces 'superoxidized' [4 Fe–4 S]$^{3+}$ clusters which give the observed $g = 2.01$ electron paramagnetic resonance (EPR) signal which is somewhat similar to that obtained from oxidized *Chromatium* HiPIP [3]. However, a $g = 2.01$ EPR signal is also associated with the [3 Fe–3 S] clusters which have now been identified in a number of proteins [4–7]. Recent magnetic circular dichroism measurements indicate that the oxidative treatment of *C. pasteurianum* Fd results in the formation of [3 Fe–3 S] clusters [8]. There is also clear evidence for the interconversion of [3 Fe–3 S] and [4 Fe–4 S] clusters in *Desulfovibrio gigas* Fd [9,10], which is known to exist in different oligomeric forms containing clusters of the two types. Mössbauer spectroscopy is a particularly useful technique for studying the relationship between the [4 Fe–4 S] and [3 Fe–3 S] clusters as their spectra are distinctively different [3,4,6]. The present investigation involves *Bacillus stearothermophilus* ferredoxin which contains a single [4 Fe–4 S]$^{2+,1+}$ cluster and has only 4 cysteine residues. The Mössbauer data presented below clearly indicate that following treatment of this protein with $K_3Fe(CN)_6$ a [3 Fe–3 S] cluster is formed.

2. Materials and methods

Bacillus stearothermophilus Fd was purified and reconstituted with ^{57}Fe as in [11]. The ferricyanide-oxidized state was obtained by titration with oxygen-free $K_3Fe(CN)_6$. The sample used was at 0.078 mM concentration in anaerobic 100 mM NaCl/100 mM Hepes buffer (pH 7.5) and was kept under an argon atmosphere. The reaction was relatively slow, requiring ~30 min at 20°C, further treatment leading to an EPR-silent form. Because of the sensitivity of *B. stearothermophilus* Fd to destruction by $K_3Fe(CN)_6$ at >0.1 mM the oxidant was added in small amounts and the reaction monitored by measuring the absorption at 420 nm. Samples were also taken at intervals and frozen for EPR measurements. When the intensity of the $g = 2.01$ signal reached a maximum the protein was put on ice and rapidly concentrated by passing through a small column of DEAE-cellulose. Reduction was carried out anaerobically by addition of sodium dithionite solution to give 2 mM final conc. The details of the Mössbauer spectroscopy were as in [11].

3. Results and discussion

The Mössbauer spectrum of the ferricyanide-oxidized Fd at 77 K (fig.1a) consists of a broadened quadrupole-split doublet which was fitted to 2 components. The major component, corresponding to ~80% of the total spectral intensity, has a chemical shift of (0.24 ± 0.01) mm/s and a quadrupole splitting of (0.57 ± 0.02) mm/s, which are identical, within the

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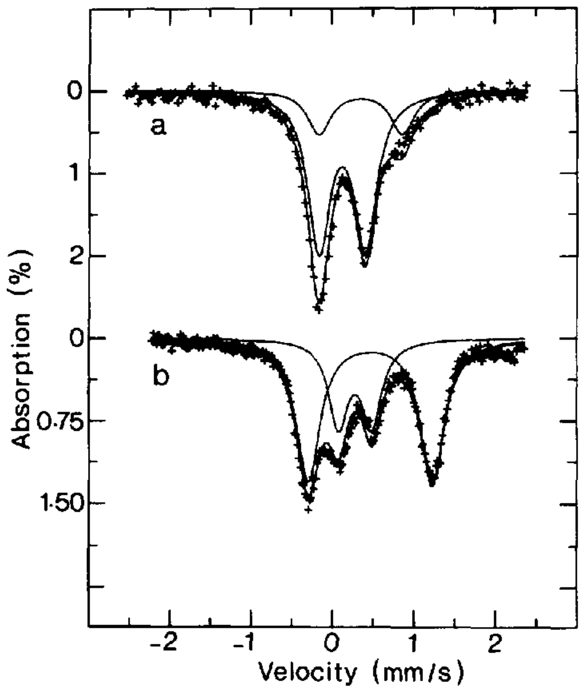

Fig.1. Mössbauer spectra of: (a) ferricyanide-oxidized *B. stearothermophilus* Fd at 77 K; (b) dithionite-reduced ferricyanide-oxidized *B. stearothermophilus* Fd at 4.2 K. The two computer-fitted components are shown together with their sum drawn through the experimental points.

experimental errors, to the values of (0.27 ± 0.03) mm/s and (0.54 ± 0.03) mm/s obtained for the [3 Fe–3 S] clusters of oxidized Fd II from *D. gigas* [6]. The values for the [4 Fe–4 S]$^{3+}$ clusters of oxidized *Chromatium* HiPIP are (0.33 ± 0.01) mm/s and (0.83 ± 0.01) mm/s [13], which are clearly substantially different. The minor component in the 77 K spectrum of the ferricyanide-oxidized Fd has parameters which are close to the average values obtained for oxidized *B. stearothermophilus* Fd at 77 K [12], which indicates that a small proportion of the [4 Fe–4 S] clusters may remain unchanged following the ferricyanide treatment.

When the ferricyanide-oxidized Fd is reduced with sodium dithionite the spectra show 2 dominant quadrupole-split doublet components. This is shown clearly in fig.1b, which is the spectrum obtained at 4.2 K. At this temperature the Mössbauer parameters of the 2 components are (0.48 ± 0.01) and (0.29 ± 0.01) mm/s for the chemical shifts and (1.52 ± 0.02) and (0.41 ± 0.02) mm/s for the quadrupole splittings. The intensity ratio of the two components is very close to 2:1. These data are again essentially identical to those obtained for the [3 Fe–3 S] clusters in reduced *D. gigas* Fd II [6], for which the chemical shifts of the 2 components are (0.46 ± 0.02) and (0.30 ± 0.02) mm/s and the quadrupole splittings are (1.47 ± 0.08) and (0.47 ± 0.02) mm/s. Both of these components have quite different parameters than those obtained from a [4 Fe–4 S] cluster in any of its 3 possible oxidation levels. The spectral component due to any unchanged [4 Fe–4 S] clusters is a broad band of absorption in this state at 4.2 K, as a result of magnetic hyperfine interactions, and therefore has negligible amplitude.

The data discussed above give a very clear indication of the conversion of the [4 Fe–4 S] clusters in *B. stearothermophilus* Fd into [3 Fe–3 S] clusters. The close similarity between the spectra shown in fig.1 and the corresponding spectra of oxidized and reduced *D. gigas* Fd II [6] provides striking evidence for this conversion. The 2:1 ratio of the two components in the reduced spectrum (fig.1b), as observed in the present work and in the case of *D. gigas* Fd II, is an important diagnostic for the presence of 3 iron atoms within the iron–sulphur cluster.

Further confirmatory evidence for the existence of [3 Fe–3 S] clusters in the ferricyanide-treated samples of *B. stearothermophilus* Fd comes from their spectra at low temperatures in the presence of applied magnetic fields, which show magnetic hyperfine splitting.

Fig.2 shows the spectrum of ferricyanide-oxidized Fd at 1.3 K in a small magnetic field. The spectrum of oxidized *D. gigas* Fd II obtained under essentially identical conditions requires three components of equal intensity in order to obtain a satisfactory computer fit [6]. These 3 components differ from each other primarily by the magnitude and anisotropy of the *A*-values which characterize their magnetic hyperfine interaction. The situation for the [3 Fe–3 S] cluster in *A. vinelandii* Fd is essentially the same although the actual *A*-values are somewhat different [5] and in both cases the fitted *A*-values may not be unique. The stick spectra labelled 1, 2 and 3 correspond to the main features of the 3 components of the fit to the spectrum of oxidized *D. gigas* Fd II under these conditions [6] while the stick spectrum labelled '4' represents any unchanged [4 Fe–4 S]$^{2+}$ clusters [12] and corresponds to ~20% of the total intensity. Bearing in mind that the lines associated with the main features of components 1, 2 and 3 are

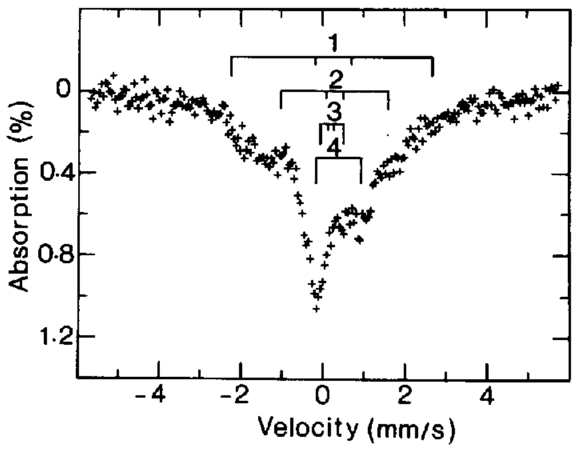

Fig.2. The Mössbauer spectrum of ferricyanide-oxidized *B. stearothermophilus* Fd at 1.3 K in a magnetic field of 0.007 T applied parallel to the γ-ray beam. The stick spectra labelled 1, 2 and 3 correspond to the 3 components of the equivalent spectrum of oxidized *D. gigas* Fd II while the stick spectrum labelled '4' corresponds to the equivalent spectrum of oxidized *B. stearothermophilus* Fd.

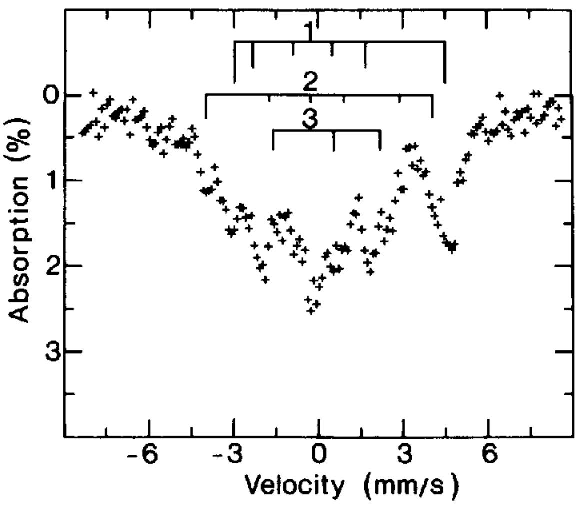

Fig.3. The Mössbauer spectra of dithionite-reduced ferricyanide-oxidized *B. stearothermophilus* Fd at 4.2 K in a magnetic field of 1.0 T applied parallel to the γ-ray beam. The stick spectra labelled '1' and '2' correspond to the two components of the equivalent spectrum of reduced *D. gigas* Fd II while the stick spectrum labelled '3' corresponds to the equivalent spectrum of reduced *B. stearothermophilus* Fd.

quite broad (~1 mm/s) and that the total splitting (corresponding to the *A*-values) may be somewhat different in this case, the stick spectra provide a good interpretation of the main features of this spectrum. A [4 Fe–4 S]$^{3+}$ cluster, equivalent to oxidized *Chromatium* HiPIP, would give a much broader flatter band of absorption [14] and in particular would not lead to the pronounced feature at ~–0.2 mm/s which can be interpreted as the superposition of lines from the spectra of [3 Fe–3 S] clusters and unconverted [4 Fe–4 S]$^{2+}$ clusters.

Fig.3 shows the spectrum obtained at 4.2 K, in a magnetic field of 1 T, from the ferricyanide-treated sample following reduction with dithionite. The equivalent spectrum of reduced *D. gigas* Fd II has been fitted with 2 components with an intensity ratio of 2:1 [6] and the stick spectra labelled '1' and '2' correspond to the main features of these components. The stick spectrum labelled '3' represents [4 Fe–4 S]$^{1+}$ clusters under these conditions [12]. Despite the relatively poor resolution of the experimental spectrum the stick spectra do provide a reasonable interpretation of the data. The experimental spectrum is clearly consistent with absorption from <–4 to >+4 mm/s as in the spectrum of the [3 Fe–3 S] cluster of reduced *D. gigas* Fd II [6] rather than with the narrower band of absorption from around –2 to +3 mm/s as would be expected if the spectrum were arising solely from clusters of the [4 Fe–4 S]$^{1+}$ type as in reduced *B. stearothermophilus* Fd.

The spectra showing magnetic hyperfine splitting are in all cases consistent with the idea that ferricyanide treatment leads to a conversion of the [4 Fe–4 S] clusters of *B. stearothermophilus* Fd into [3 Fe–3 S] clusters rather than into a [4 Fe–4 S]$^{3+}$ superoxidized state. This gives valuable confirmation of this conversion in addition to the clear evidence provided by the non-magnetic Mössbauer spectra discussed earlier.

Acknowledgements

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References

[1] Carter, C. W. (1977) in: Iron–Sulfur Proteins (Lovenberg, W. ed) vol. 3, pp. 158–204, Academic Press, New York.

[2] Sweeney, W. V., Bearden, A. J. and Rabinowitz, J. C. (1974) Biochem. Biophys. Res. Commun. 59, 188–194.

[3] Cammack, R., Dickson, D. P. E. and Johnson, C. E. (1977) in: Iron–Sulfur Proteins (Lovenberg, W. ed) vol. 3, pp. 283–330, Academic Press, New York.

[4] Emptage, M. H., Kent, T. A., Huynh, B. H., Rawlings, J., Orme-Johnson, W. H. and Münck, E. (1980) J. Biol. Chem. 255, 1793–1796.

[5] Stout, C. D., Ghosh, D., Pattabhi, V. and Robbins, A. H. (1980) J. Biol. Chem. 255, 1797–1800.

[6] Huynh, B. H., Moura, J. J. G., Moura, I., Kent, T. A., LeGall, J., Xavier, A. V. and Münck, E. (1980) J. Biol. Chem. 255, 3242–3244.

[7] Kent, T. A., Dreyer, J.-L., Emptage, M. H., Moura, I., Moura, J. J. G., Huynh, B. H., Xavier, A. V., LeGall, J., Beinert, H., Orme-Johnson, W. H. and Münck, E. (1981) in: Symposium on Interactions between Iron and Proteins in Oxygen and Electron Transport (Ho, C. ed) Elsevier Biomedical, Amsterdam, New York, in press.

[8] Thomson, A. J., Robinson, A. E., Johnson, M. K., Cammack, R., Rao, K. K. and Hall, D. O. (1981) Biochim. Biophys. Acta 637, 423–432.

[9] Kent, T. A., Moura, I., Moura, J. J. G., Lipscomb, J. D., Huynh, B. H., LeGall, J., Xavier, A. V. and Münck, E. (1982) FEBS Lett. 138, 55–58.

[10] Moura, J. J. G., Moura, I., Kent, T. A., Lipscomb, J. D., Huynh, B. H., LeGall, J., Xavier, A. V. and Münck, E. (1982) J. Biol. Chem. in press.

[11] Mullinger, R. N., Cammack, R., Rao, K. K., Hall, D. O., Dickson, D. P. E., Johnson, C. E., Rush, J. D. and Simopoulos, A. (1975) Biochem. J. 151, 75–83.

[12] Middleton, P., Dickson, D. P. E., Johnson, C. E. and Rush, J. D. (1978) Eur. J. Biochem. 88, 135–141.

[13] Middleton, P., Dickson, D. P. E., Johnson, C. E. and Rush, J. D. (1980) Eur. J. Biochem. 104, 289–296.

[14] Dickson, D. P. E., Johnson, C. E., Cammack, R., Evans, M. C. W., Hall, D. O. and Rao, K. K. (1974) Biochem. J. 139, 105–108.