CONVERSION OF [3 Fe–3 S] INTO [4 Fe–4 S] CLUSTERS IN A *DESULFOVIBRIO GIGAS* FERREDOXIN AND ISOTOPIC LABELING OF IRON–SULFUR CLUSTER SUBSITES

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1. Introduction

Desulfovibrio gigas ferredoxin is isolated in different oligomeric forms. The oligomers are assembled from a single type of polypeptide, each monomer having 57 amino acids of known sequence [1]. It has been suggested from studies with cell extracts that the tetrameric form, Fd II, mediates electron transport between cytochrome c_3 and the sulfite reductase system and that the trimeric Fd I serves as an electron carrier in the phosphoroclastic reaction [2]. These studies have also demonstrated that Fd II stimulates the phosphoroclastic reaction, after a long time lag, in the presence of pyruvate [2]. It has been shown by Mössbauer spectroscopy that Fd II contains 1 [3 Fe–3 S] cluster/monomer [3]. On the other hand, in Fd I both [3 Fe–3 S] and [4 Fe–4 S] clusters are present [1,3], probably in variable proportions. Although both oligomers are capable of performing different physiological tasks, one wonders whether both are biologically active and whether the 2 cluster types interconvert. These questions have been brought into sharper focus by 3 recent observations.

(i) Studies in cell extracts have shown that Fd II can develop EPR signals typical of reduced [4 Fe–4 S] clusters [4].

(ii) The [4 Fe–4 S] clusters of a *Clostridium pasteurianum* ferredoxin can be converted into [3 Fe–3 S] clusters upon treatment with ferricyanide [5]. We have obtained similar results with *D. gigas* ferredoxin [6].

(iii) Mössbauer spectroscopy has shown that a [3 Fe–3 S] cluster is converted into a [4 Fe–4 S] structure when beef-heart aconitase is activated with ferrous ions [7].

Using *D. gigas* Fd II we have studied iron–sulfur cluster reconstitutions and conversions with Mössbauer and electron paramagnetic resonance (EPR) spectroscopy. Here we demonstrate that the [3 Fe–3 S] cluster of Fd II is converted into a [4 Fe–4 S] cluster by incubation of the protein with Fe^{2+} in the presence of dithiothreitol.

By using 95% enriched ^{57}Fe for the incubation procedure, we have found that the iron is incorporated into 1 (or possibly 2) subsites of the newly formed [4 Fe–4 S] core. We report here studies of the reduced form of these [4 Fe–4 S] clusters. Mössbauer studies of the oxidized form, as well as cluster reconstitutions and studies of the time course of the cluster interconversions, are dealt with in [6].

2. Materials and methods

Desulfovibrio gigas Fd II was isolated as in [8]. The Mössbauer sample, subsequently referred to as sample A, was prepared as follows: Fd II, 1.4 mM monomer, was reduced under anaerobic conditions with a stoichiometric amount of $Na_2S_2O_4$. After addition of a 5-fold excess of dithiothreitol and a 4-fold excess of both Fe^{2+} (95% enriched in ^{57}Fe) and sodium sulfide the sample was incubated anaerobically for 6 h at 25°C, exposed to air, and then passed through a Sephadex G-25 column to remove the

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reagents. The protein was then repurified on a DEAE-cellulose column. After passing the sample through a second Sephadex column, it was concentrated by evaporation under argon. The sample used for EPR was incubated similarly and then frozen in an EPR tube without any further purification. All samples were buffered in 50 mM Tris–HCl at pH 7.6.

A sample of a *D. gigas* ferredoxin containing a [4 Fe–4 S] cluster enriched in ^{57}Fe was prepared from apoprotein by a chemical reconstitution procedure, similar to that in [9]. This material, referred to as 'reconstituted Fd', was used here for spectral comparisons (see [6]).

EPR spectra were recorded with a Varian E-109 spectrometer fitted with an Oxford Instruments ESR-10 continuous flow cryostat. The Mössbauer spectra were recorded with a constant acceleration spectrometer. Isomer shifts, δ Fe, are quoted relative to Fe metal at room temperature.

3. Results

We have prepared samples of ^{57}Fe-reconstituted Fd using apoprotein derived from Fd II. Mössbauer and EPR studies [6] showed that this material contains a [4 Fe–4 S] cluster. In the reduced state the reconstituted protein yields an EPR spectrum (see fig.8 of [1]) identical to that shown in fig.1. The observed g-values at $g = 2.07$, 1.94 and 1.91 are the same as those observed for the [4 Fe–4 S]$^{1+}$ cluster of Fd I. The EPR spectra also contain a minority species with features at $g = 2.04$ and 1.86. The latter signals are also observed when Fd I is reduced in the presence of dimethylsulfoxide (>40%, by vol.) suggesting that they arise from partially unfolded protein. Mössbauer spectra of reduced reconstituted Fd are shown in fig.2,3b (hatch marks). The spectrum taken at 4.2 K is very similar to that [10] observed for the [4 Fe–4 S] ferredoxin from *Bacillus stearothermophilus* (the spectra are compared in fig.9a of [1]). At 90 K the relaxation rate of the electronic spin $S = \frac{1}{2}$ is fast and quadrupole doublets are observed. One can discern, albeit with poor resolution, 2 doublets, labeled I and II. A spectral decomposition [6] indicates that both doublets have about equal intensity, suggesting that the type-I sites (yielding doublet I) and type-II sites are each occupied by 2 iron atoms.

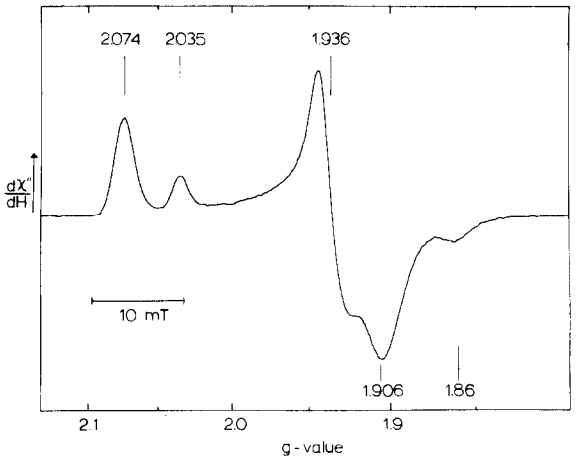

Fig.1. EPR spectrum of a Fd II sample incubated in the EPR tube for 1 h with excess amounts of dithionite, dithiothreitol, ferrous ion, and sulfide. The iron added for the incubation had ^{57}Fe in natural abundance: temperature, 8 K; microwave power, 0.03 mW; modulation amplitude, 4 g; microwave frequency, 9.2 GHz.

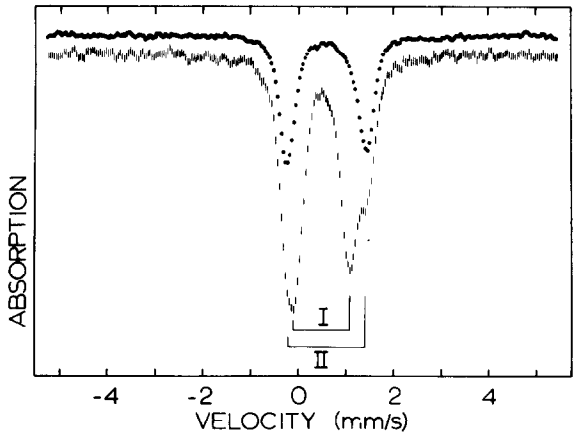

Fig.2. Mössbauer spectra of reduced reconstituted Fd (hatch marks) and sample A (Fd II incubated with ^{57}Fe, sulfide and dithiothreitol). The spectra were recorded at 90 K in zero magnetic field. Doublets I and II observed for reconstituted Fd are indicated by brackets.

The [3 Fe–3 S] cluster of Fd II yields an EPR signal at $g = 2.01$ in the oxidized state; the reduced cluster is EPR-silent [3]. After incubation with Fe^{2+} the reduced sample yielded the EPR spectrum shown in fig.1. The development of an EPR signal with features identical to those of reconstituted Fd leaves little doubt that [4 Fe–4 S] clusters have been formed. Quantitation of the EPR spectrum relative to the $g = 2.01$ signal of the starting material showed that 72% of the [3 Fe–3 S] clusters had been converted into the $g = 1.94$ species.

For the discussion of the Mössbauer spectra the

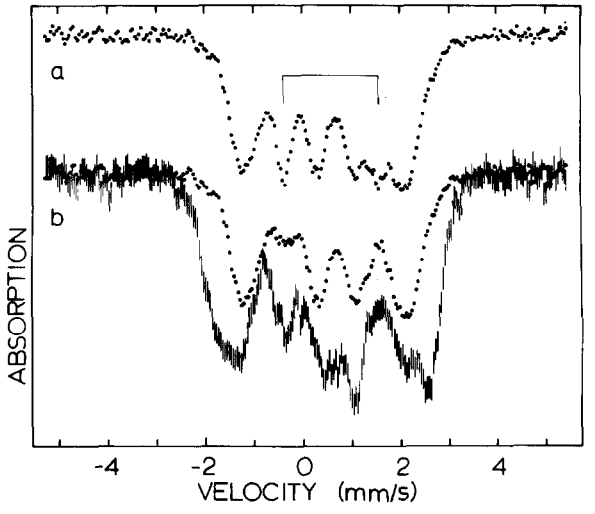

Fig.3. Mössbauer spectra recorded at 4.2 K in magnetic fields of 60 mT applied parallel to the observed γ-radiation. The spectrum of sample A in (a) contains a quadrupole doublet indicated by brackets (see footnote). The spectrum of reconstituted Fd shown in (b) (hatch marks) was prepared by subtracting from the raw data contributions from a reduced [3 Fe–3 S] cluster (10%) and a ferrous impurity (5%). Similar subtractions were performed to generate the spectrum of reconstituted Fd shown in fig.2. Also shown in (b) (•) is a spectrum of sample A prepared by subtracting the doublet (15% of ^{57}Fe) indicated in (a).

reader is reminded that sample A was incubated with 95% enriched ^{57}Fe. If [4 Fe–4 S] clusters are formed by addition of a single ^{57}Fe to a [3 Fe–3 S] cluster one will observe only Mössbauer spectra of certain subsites of the converted clusters. Unconverted [3 Fe–3 S] clusters contain ^{57}Fe only in natural abundance (2.2%) and practically do not contribute to the spectra.

The Mössbauer spectrum of sample A (fig.2,•) recorded at 90 K consists of a single doublet with quadrupole splitting ΔE_Q = 1.67 mm/s and isomer shift δ_{Fe} = 0.60 mm/s. This doublet corresponds to doublet II of reconstituted Fd, id., the ^{57}Fe has been incorporated into a type-II site; the type-I sites are unoccupied. In order to determine sign (ΔE_Q) and the asymmetry parameter η of the quadrupole interaction we have studied the sample at 90 K in an applied field of 4 T. A good fit to the spectrum was obtained by assuming that only one species, with $\Delta E_Q > 0$ and $\eta = 0$, was present. Thus the 90 K spectra of sample A do not reveal whether 1 or 2 nuclei/cluster contribute to doublet II. (Actually, if exchange among the type-II sites would take place during incubation, fractional occupancies would need to be considered. As can be seen from the spectra shown in fig.2 there is no evidence for exchange between the type-I and type-II sites.)

As pointed out above the EPR spectra of reduced sample A and reduced reconstituted Fd are identical. With this information it is instructive to compare the low temperature Mössbauer spectra. Since the electronic spin relaxation rate of [4 Fe–4 S]$^{1+}$ clusters is slow at 4.2 K the spectra will exhibit paramagnetic hyperfine structure. The spectrum of reduced reconstituted Fd shown in fig.3b (hatch marks) consists, in principle, of 4 magnetic subspectra, one resulting from each of the 4 cluster subsites. The corresponding spectrum of sample A, shown also in fig.3b (•) has markedly different features*. In particular, the overall magnetic splitting observed for sample A is smaller than that observed for reconstituted Fd. This observation leads to the conclusion that the internal magnetic fields, H_{int}, at type-II site nuclei are smaller in magnitude than those associated with type-I sites. Mössbauer studies [10] of the *B. stearothermophilus* ferredoxin in strong applied fields have demonstrated that 2 nuclei of the [4 Fe–4 S] cluster experience a positive H_{int} (a consequence of spin coupling), while $H_{int} < 0$ for the remaining sites. High-field spectra of sample A taken at 4.2 K reveal that the type-II sites have $H_{int} > 0$. Preliminary analyses of the high field data suggest that they can be fitted by assuming that only one spectral component is present. This would imply that the ^{57}Fe has been incorporated either into 1 subsite or into 2 highly equivalent subsites. Experiments to resolve this question are in progress.

We have prepared a sample by incubating Fd II with iron 99.9% enriched in the Mössbauer-silent isotope ^{56}Fe. This procedure allowed us to follow the fate of the iron present in the original 3-Fe cluster. A Mössbauer study showed that the spectra of the intrinsic iron of Fd II reflect the new electronic environments resulting from the cluster transformation.

* The 4.2 K spectrum of sample A is shown in fig.3a. Studies in strong applied magnetic fields and in zero field revealed that it contains, in addition to the major magnetic component, a quadrupole doublet accounting for 15% of total ^{57}Fe. The doublet, indicated by the bracket, has parameters identical to those observed for the 90 K spectrum of sample A. The spectrum of sample A shown in fig.3b was prepared by subtracting the doublet from the raw data. We argue elsewhere [6] that the doublet belongs to a subpopulation of molecules with fast electronic relaxation, probably caused by aggregation

4. Discussion

We have shown here that the [3 Fe–3 S] clusters of Fd II are converted into [4 Fe–4 S] core structures when the protein is incubated with Fe^{2+} and S^{2-} in the presence of dithiothreitol. Cluster conversion has been demonstrated by the appearance of substantial $g = 1.94$ signals identical to those observed for the [4 Fe–4 S] clusters of Fd I and reconstituted Fd. This is supported by the observation that the Mössbauer spectra of the reduced, EPR-active sample have features typical of $[4\ Fe–4\ S]^{1+}$ clusters. The evidence presented here is corroborated by studies of sample A in the oxidized state [6]: the observed diamagnetism and the values found for the Mössbauer parameters, in particular $\delta Fe = 0.45$ mm/s, strongly suggest the presence of a $[4\ Fe–4\ S]^{2+}$ cluster. ([3 Fe–3 S] clusters are paramagnetic in all known states.)

We have also demonstrated here isotopic labeling of specific cluster subsites. This allows us to study in considerable detail the hyperfine interactions of the [4 Fe–4 S] core structures. Moreover, this technique will allow us to correlate spectra of subsites in different oxidation states.

Recent studies have shown that [4 Fe–4 S] clusters can be converted into [3 Fe–3 S] centers by either ferricyanide oxidation [5,6] or by reduction with dithionite at high ionic strength [6]. Using procedures such as used here for the *D. gigas* protein, it seems feasible to rebuild [4 Fe–4 S] clusters with incorporation of ^{57}Fe labels. If this technique could be applied to a single cluster of an enzyme with multiple clusters (hydrogenase comes to mind) spectral assignments would be greatly facilitated. Finally, it would be interesting to explore whether cubane $[MoFe_3–4\ S]$ clusters can be formed by replacing iron by molybdenum in the incubation medium; such clusters are of interest in nitrogenase research.

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References

[1] Xavier, A. V., Moura, J. J. G. and Moura, I. (1981) Struct. Bond. 43, 187–213.
[2] Moura, J. J. G., Xavier, A. V., Hatchikian, C. E. and LeGall, J. (1978) FEBS Lett. 89, 177–179.
[3] 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.
[4] Moura, J. J. G., LeGall, J. and Xavier, A. V. (1982) submitted.
[5] 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.
[6] Moura, J. J. G., Moura, I., Kent, T. A., Lipscomb, J. D., Huynh, B. H., LeGall, J., Xavier, A. V. and Münck, E. (1982) submitted.
[7] Kent, T. A., Dreyer, J.-L., Kennedy, M. C., Huynh, B. H., Emptage, M. H., Beinert, H. and Münck, E. (1982) Proc. Natl. Acad. Sci. USA in press.
[8] Bruschi, M., Hatchikian, C. E., LeGall, J., Moura, J. J. G. and Xavier, A. V. (1976) Biochim. Biophys. Acta 449, 275–284.
[9] Hong, J.-S. and Rabinowitz, J. C. (1970) J. Biol. Chem. 245, 6574–6581.
[10] Middleton, P., Dickson, D. P. E., Johnson, C. E. and Rush, J. D. (1978) Eur. J. Biochem. 88, 135–141.