Volume 332, number 3, 268–272 FEBS 13117 October 1993

Selective interaction of ferricyanide with cluster I of *Clostridium pasteurianum* 2[Fe_4S_4] ferredoxin

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Received 30 August 1993

Treatment of *Clostridium pasteurianum* ferredoxin (CpFd) with stoichiometric amounts of potassium ferricyanide results in the specific conversion of cluster I into a $Fe_3S_4^+$ species while leaving cluster II unaltered. Ferricyanide-treated CpFd derivative has been purified and characterized through biochemical and spectroscopical techniques. The cluster conversion process is reversible and reconstitution of native CpFd has been afforded under appropriate conditions.

Ferredoxin; NMR; *Clostridium pasteurianum*

1. INTRODUCTION

Clostridium pasteurianum ferredoxin is a small iron-sulfur protein (55 AA, MW 6,000) containing two Fe_4S_4 clusters [1–3]. The two paramagnetic clusters generate a characteristic ^1H NMR spectrum with several, relatively narrow isotropically shifted signals in the downfield region [4]. Recently, through 1D and 2D NMR techniques, it has been possible to identify and assign pairwise the hyperfine shifted signals to the β-CH_2 protons of the eight cluster-coordinated cysteines [4–7]. In addition, 2D exchange correlation experiments allowed us to attribute the individual resonances to either cluster I or cluster II, taking advantage of the small redox potential difference existing between the clusters [8]. The ^1H NMR spectral features of either cluster show a marked internal symmetry reflecting the deep structural equivalence of the two Fe_4S_4 groups revealed by crystallographic studies [9,10]. In the context of a larger project aiming at the analysis of the structure–function relationships in iron-sulfur proteins, we decided to investigate whether the two clusters of *Clostridium pasteurianum* ferredoxin might exhibit some difference in reactivity in spite of their strict structural similarity. It was previously reported that the two clusters are characterized by an inequivalent distribution of fixed electric charges, most of the negative charges being localized around cluster II whereas cluster I lies in an essentially uncharged environment [11]. We thought that the different electrostatic environment of the two clusters could originate a different pattern of reactivity toward charged species. Starting from these considerations, we decided to investigate the interaction of oxidized *Clostridium pasteurianum* ferredoxin with ferricyanide through ^1H NMR spectroscopy. The reaction with ferricyanide is of particular interest for iron-sulfur proteins and related model systems since it often affords conversion of the Fe_4S_4 clusters into the corresponding Fe_3S_4 species [12–15]. Preliminary studies on the reaction of CpFd with ferricyanide had been reported by Thomson et al. and by Spiro et al. [12–14] and the resulting derivative was characterized through MCD, EPR and resonance Raman techniques. Their results strongly suggested the conversion of Fe_4S_4 clusters to Fe_3S_4 moieties. Furthermore, Spiro et al. measured the intensity of the EPR signal of the treated species and found that it corresponded to maximally 0.80 spin/molecule, thus suggesting that the ferricyanide-titrated protein contained only one Fe_3S_4 cluster [13,14]. No reconstitution procedure was, however, reported.

Since cluster assignment through ^1H NMR spectroscopy is now available [8], we decided to further investigate, mainly through this technique this system in order to obtain further and more detailed information on the CpFd–ferricyanide derivative.

2. EXPERIMENTAL

Clostridium pasteurianum was grown and ferredoxin isolated and purified according to the method of Rabinowitz [16]. The purity of the sample was checked by absorption spectroscopy monitoring the A_{390}/A_{280} absorbance ratio. For ^1H NMR experiments the protein was dissolved in 50 mM P_i buffer, pH 8.5, containing 0.8 M NaCl. Deuteration of the sample was achieved by utilizing an ultrafiltration Amicon cell, equipped with a YM5 membrane. At least five changes of deuterated buffer were performed to ensure satisfactory solvent exchange. The pH values are reported as uncorrected pH meter readings.

The ferricyanide-treated samples have been purified by passing

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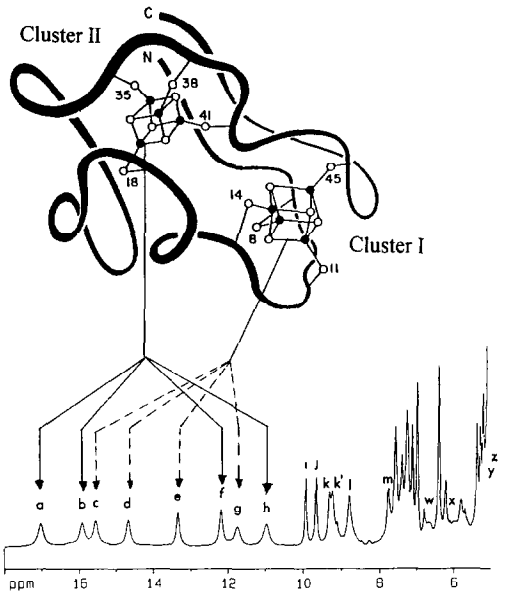

Fig. 1. ^{1}H NMR spectrum of oxidized *Clostridium pasteurianum* ferredoxin. The hyperfine shifted resonances are labeled as previously reported. A schematic drawing of the protein and the two Fe4S4 clusters is shown.

them on a small Sephadex G-25 column (10 × 1 cm) equilibrated with 50 mM phosphate buffer, pH 8.5 to eliminate the excess ferricyanide and the Prussian blue produced by reaction. Then, the protein was absorbed on a short DE-52 column (5 × 1 cm) equilibrated with the same buffer, washed with 0.2 M NaCl and eluted with 0.8 M NaCl.

The purified ferricyanide-treated protein has been reconstituted by incubating dithionite-reduced protein samples (0.5–1.0 mM) for 45–60 min, under anaerobic conditions, with 1,4-dithiothreitol (5 mM) and $FeSO_4$ (5 mM) solution in 50 mM phosphate and 0.8 M NaCl. The reconstituted ferredoxin has been purified as in the case of the ferricyanide-treated sample.

The ^{1}H NMR spectra were recorded on both an AMX 600 and an MSL 200 Bruker spectrometer. Chemical shift values are referred to DSS.

The CD spectra in the visible region were performed on a Jasco J500C spectropolarimeter operating at room temperature.

The iron-content determination was performed spectrophotometrically using the *o*-phenanthroline method [17–19]. Protein concentration was determined by UV spectroscopy and utilizing the Bradford method [20–22]. We obtained an average metal/protein ratio of about 6.7 ± 0.5 for the purified ferricyanide treated species and 8.2 ± 0.5 for the native protein.

3. RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of oxidized CpFd is shown in Fig. 1. The spectrum is characterized by several isotropically shifted signals in the range 20–10 ppm. Recent 2D NMR studies have permitted the identification of the geminal connectivities of the hyperfine shifted signals and their assignment to the β-CH_2 protons of the cluster-coordinated cysteines. In particular signal pairs ***c–k***, ***d–z***, ***e–w*** and ***g–l*** were assigned to the eight β-CH_2 protons of the four cysteines bound to cluster I, namely Cys8, 11, 14 and 47, whereas signal pairs ***a–k′***, ***b–y***, ***f–x*** and ***h–m*** were assigned to the cysteines bound to cluster II, namely Cys18, 37, 40 and 43. The complete sequence specific assignment of the Cys β-CH_2 protons is available [23].

The ^{1}H NMR titration of oxidized CpFd ferredoxin with increasing amounts of potassium ferricyanide was carried out at 298 K, pH 8.5, 50 mM phosphate buffer, 0.8 M NaCl. From the behavior of the hyperfine shifted

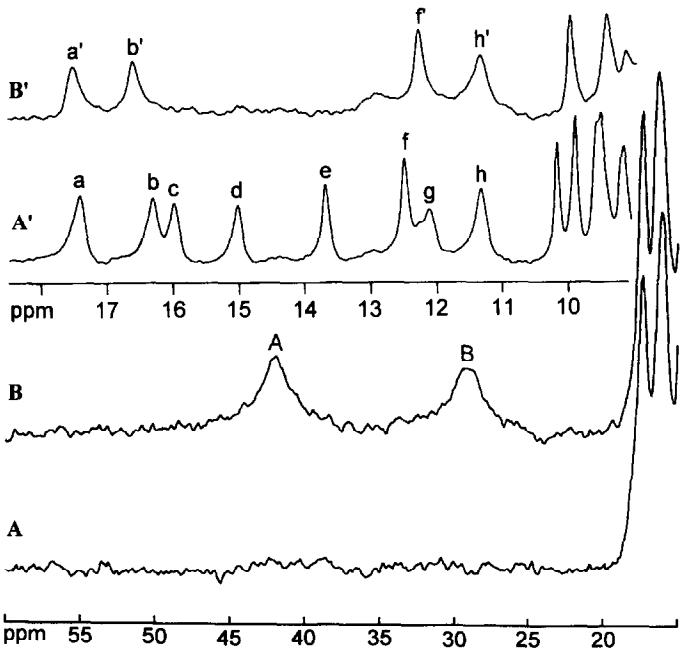

Fig. 2. ^{1}H NMR titration of *Clostridium pasteurianum* ferredoxin before (A) and after (B) treatment with potassium ferricyanide. In A′ and B′ the detail of the 20–10 ppm region is shown (A′ is native CpFd, B′ is the ferricyanide-treated species).

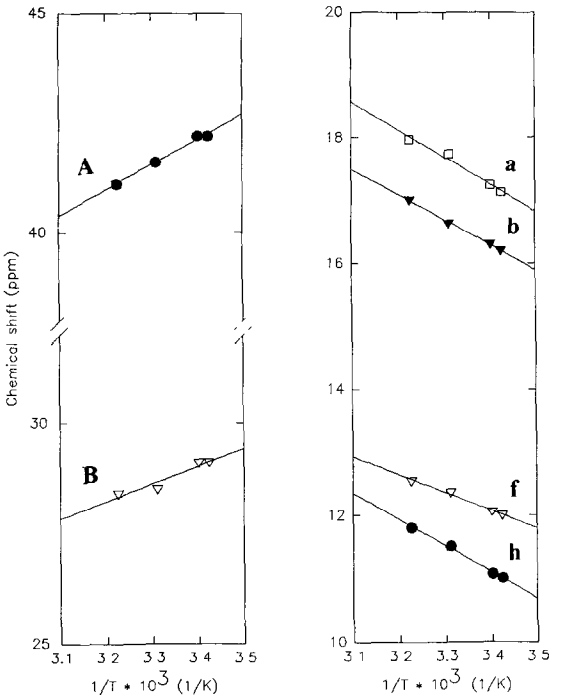

Fig. 3. Temperature dependence of the hyperfine shifted signals of ferricyanide-treated CpFd.

signals it appears that ferricyanide reacts with the protein in a specific and selective way. The observed spectral changes are indicative of the progressive transformation of oxidized *Clostridium pasteurianum* ferredoxin into a new species, under slow exchange conditions. The ^{1}H NMR spectrum of the new species is characterized by the appearance of at least two broad signals with larger downfield isotropic shifts (signals A and B), and by the progressive disappearance of some resonances in the 20–10 ppm region (Fig. 2A). Complete conversion of ferredoxin into the new species is achieved at ferricyanide/ferredoxin ratios lower than 2 in high ionic strength i.e. 0.8 M NaCl. We have observed a marked influence of the ionic strength on the course of the reaction. In fact, if the reaction is carried out at 0.1 M NaCl, instead of 0.8 M, complete conversion of oxidized CpFd into the new species is obtained only for ferricyanide/ferredoxin ratios greater than 12. Yet, such large amounts of ferricyanide also cause extensive protein denaturation. The new species was purified through gel filtration and its iron content, determined by the *o*-phenanthroline method, resulted to be 6.7 ± 0.5 ions per mole of protein.

A close inspection of the ^{1}H NMR spectrum of the new species in the 20–10 ppm region reveals only four hyperfine shifted signals instead of the eight observed in the native protein (Fig. 2B). Out of these two signals (***a′*** and ***f′***) are exactly in the same position of signals ***a*** and ***f*** of native ferredoxin whereas signals ***b′*** and ***h′*** are only slightly shifted with respect to signals ***b*** and ***h***. On the other hand, signals ***c***, ***d***, ***e***, and ***g*** completely disappear upon ferricyanide treatment. Since we know that signals ***a***, ***b***, ***f***, and ***h*** belong to cluster II, and signals ***c***, ***d***, ***e***, and ***g*** to cluster I, it is straightforward to interpret the present results in terms of ferricyanide specifically reacting with cluster I. Thus, oxidation with ferricyanide would cause cluster I to transform into a new species characterized by broad hyperfine signals with larger isotropic shifts whereas cluster II is not significantly perturbed as witnessed by the very modest chemical shift changes of its signals. The small changes observed for resonance ***b***, assigned to one β-CH_2 proton of Cys43 [23], could be due to the vicinity of such cysteine to cluster I which has reacted with ferricyanide. The change of the electronic properties of cluster I is therefore sensed by the nearby Cys43 residue.

On the basis of previous experiments on the present and analogous iron-sulfur systems, it is tempting to hypothesize that the reason for the drastic spectral changes in cluster I is the extraction of one of its iron ions and subsequent conversion into a Fe_3S_4 cluster. The ^{1}H NMR spectral features of the new cluster are indeed resemblant of those of previously reported ^{1}H NMR spectra of Fe_3S_4 species [24,25].

In Fig. 3 the temperature dependence of the ^{1}H NMR signals of the purified CpFd sample, in the temperature interval 292–310 K, are reported. It is apparent that signals belonging to the unmodified cluster II retain their characteristic anti-Curie dependence profile [8], whereas signal A and B belonging to the modified cluster I now exhibit a Curie type behavior. A similar temperature dependence profile has recently been reported by Moura et al. for the two most downfield hyperfine shifted signals assigned to the β-CH_2 protons of Cys50 bound to the Fe_3S_4 cluster in *Desulfovibrio gigas* ferredoxin II [25].

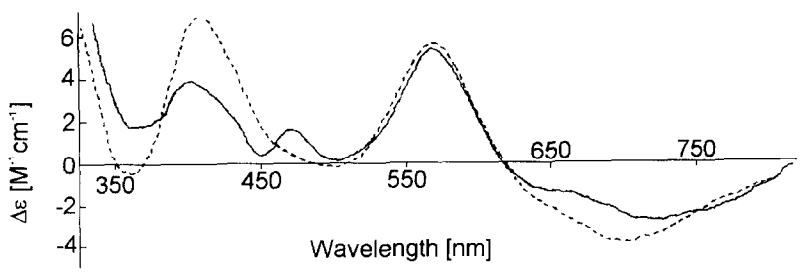

Fig. 4. CD spectra of native (A) and ferricyanide-treated (B) and reconstituted (C) CpFd.

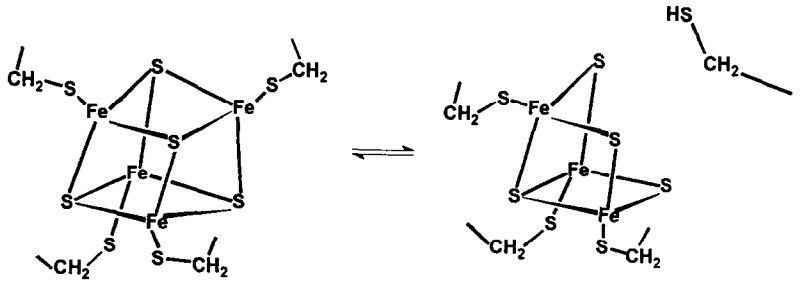

Fig. 5. Schematic drawing of the Fe_4S_4 to Fe_3S_4 cluster conversion by ferricyanide treatment.

To further characterize the ferricyanide-treated protein we employed CD spectroscopy which is indeed very sensitive to conformational changes of metal chromophores in metalloproteins. The CD spectra of native and ferricyanide-treated CpFd are reported in Fig. 4. The CD spectrum of oxidized ferredoxin is characterized by three intense positive bands, respectively, located at 310, 410 and 570 nm, plus a broad negative band at about 700 nm [26]. Treatment with ferricyanide causes drastic spectral changes such as appearance of a new positive band at 470 nm, decrease in intensity of the 410 nm transition and a marked change in the negative band at 700 nm. Similar transitions were previously observed in the CD spectrum of *Azotobacter vinelandii* ferredoxin I, containing 7 iron centers [27].

The reconstitution of 8Fe8S ferredoxin starting from the ferricyanide-treated protein has been performed following procedures commonly used to reconstitute 4Fe4S clusters starting from the respective 3Fe4S species (see section 2). The reaction has been monitored through ^1H NMR and CD spectroscopies; in both cases spectra indistinguishable from those of the native CpFd protein were obtained indicating that the process is completely reversible.

In conclusion all the reported analytical and spectroscopic data clearly indicate that the CpFd ferricyanide-treated species is characterized by the presence of two inequivalent iron-sulfur moieties cluster I being a Fe_3S_4 complex whereas cluster II maintains its original Fe_4S_4 composition.

Concerning the identity of the extracted iron ion some comments can be done owing to the availability of detailed structural information on several iron sulfur proteins. A peculiar feature of Fe_4S_4 clusters in a large number of proteins is their ability to loose one iron ion upon oxidation [28]. Typical examples are *Desulphovibrio gigas* Fd II, *Desulphovibrio africanus* Fd III, *Pyrococcus furiosus* Fd, IRE-BP mRNA binding protein as well as enzymes like aconitase and L-serine dehydratase [29–34]. Possible physiological roles of the iron ion loss/uptake mechanisms, resulting in activity control and modulation, have been suggested for such proteins. Comparing the primary sequences of these Fe_4S_4 proteins containing a labile metal we observe that the first three iron ligands (Cys, Y = Cys or Asp, and Cys) are always comprised in a sequence of the type

Cys-X-X-Y-X-X-Cys

whereas the fourth ligand (Cys) lies in a different portion of the protein [35,36]. Upon conversion of a Fe_4S_4 cluster into the corresponding Fe_3S_4 cluster it is usually the central Y residue (Cys or Asp) of the above sequence to be detached. This would suggest that in the case of CpFd Cys11 could be the one detached from the cluster upon oxidation with ferricyanide (see Fig. 5); interestingly such a cysteine results to be solvent exposed [9–11].

Acknowledgements: Dr. Roberto Monnanni and Dr. Giovanni Vallini are gratefully acknowledged for the gift of the CpFd samples.

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