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Interconversions between the 3Fe and 4Fe forms of the iron-sulfur clusters in the ferredoxin from *Thermodesulfobacterium commune*: EPR characterization and potentiometric titration

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The amount of 3Fe clusters in *Thermodesulfobacterium commune* ferredoxin is strongly dependent upon the presence of oxygen during the purification. An average of one 3Fe cluster per monomer can be found when the purification is not strictly anaerobic. These clusters are converted into |4Fe-4S| clusters by adding dithionite at usual pH and without adjunction of Fe^{2+}. The EPR potentiometric titration reveals the existence of several types of 3Fe clusters with negative midpoint potentials differing by more than 100 mV. When the |4Fe-4S| clusters are partially reduced the EPR signal is composed of two different rhombic components. The component with $g_z = 2.04$ could be related to a site implicated in the interconversion processes. In the fully reduced state, the spectrum presents the typical features of two interacting |4Fe-4S| clusters as those observed in two |4Fe-4S| bacterial ferredoxins. From the redox titration curves the midpoint potentials of these clusters are estimated at −395 and −435 mV.

Introduction

Many examples have been reported where proteins containing iron-sulfur clusters have shown their ability to accommodate 3Fe or 4Fe clusters at the same site. Typical examples which have been studied in detail are Aconitase [1], *Desulfovibrio gigas* Fd II [2], *Azotobacter vinelandii* Fd I [3], *Azotobacter chroococcum* ferredoxin [4] and *Pseudomonas ovalis* ferredoxin [5]. The change from one cluster type to an other can be obtained either by an interconversion process induced by reductive or oxidative agents or by a reconstitution of the iron sulfur centre from the apoprotein. The 4Fe-to-3Fe conversion usually requires strong oxidative agents, whereas incubation with Fe^{2+} [6,7] or high pH conditions [1,4] are necessary to induce an efficient 3Fe-to-4Fe conversion. We report in this paper an EPR study on this subject performed on the ferredoxin from the thermophilic sulfate reducing bacterium *Thermodesulfobacterium commune*. The presence of 3Fe clusters appears to be strongly correlated to the presence of oxygen during the purification procedure. The conversion of these clusters into 4Fe-4S clusters can be induced by addition of dithionite at usual pH values. We discuss the results of redox titration experiments and give an estimate of the midpoint potentials of the different types of iron-sulfur clusters which are observed in the protein.

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Material and Methods

The purification procedures and the characterization of *T. commune* ferredoxin have been previously described [8]. When precautions are taken against oxygen, a dimeric form is obtained with a molecular weight equal to 12500. For convenience, this ferredoxin will be noted Fd(an) in the following. The presence of two iron-sulfur clusters per monomer of Fd(an) was found on the basis of the iron and labile sulfur content and the values of the optical densities. Although this analysis is in favour of a large majority of |4Fe-4S| clusters the precision is not sufficient to estimate the relative amount of 3Fe clusters. This point is discussed in the present paper. When the purification is not performed in strict anaerobic conditions three different oligomeric forms can be separated which all exhibit the same aminoacid composition as Fd(an). The more acidic fraction is a trimer (M_r 21000) and was found by EPR to contain the largest proportion of 3Fe clusters. Only this trimeric fraction, which is noted Fd(a), will be considered in the following.

The EPR spectra were recorded at low temperature on a Varian E112 X-band spectrometer equipped with an Air-Product Helitran Gas-Flow System. The calibration of the spectrometer was checked with the strong pitch Varian sample placed in cavity II of the dual sample cavity. Spin quantitation was deduced from numerical double integration of the spectra using copper sulfate as a standard. The microwave power was adjusted at a level sufficiently low to avoid saturating effects.

The potentiometric titrations of Fd(an) and Fd(a) were performed in anaerobic conditions at 18°C with an Ag|AgCl electrode. The concentrations of Fd(an) and Fd(a) as measured spectrophotometrically at 385 nm, were 0.18 mM and 0.11 mM, respectively, in 0.1 M Tris-HCl at pH 7.6. The mediators used were: 2-hydroxy-1,4-naphthoquinone, phenosafranine, benzyl viologen, methyl viologen all at 8 μM concentration. The potential was adjusted by small additions of 0.025 M $Na_2S_2O_4$. After equilibration for about 10 min, 0.15 ml of the ferredoxin solution was taken and frozen in calibrated EPR tubes.

Results and Comments

Conversion of the 3Fe clusters into |4Fe-4S| clusters

In the oxydized state, the EPR spectra given by Fd(an) and Fd(a) are typical of 3Fe clusters (Fig. 1a and b) and exhibit the main characteristic features which have been reported and discussed for *A. vinelandii* Fd I and *D. gigas* Fd II [9,10]: below 20 K the shape is asymmetric and centered at $g = 2.01$. The saturation experiments performed at 6.5 K indicate a fast spin-lattice relaxation rate with a T_1 of about 6 μs compared to 3 μs for *A. vinelandii* Fd I and *D. gigas* Fd II at the same temperature [11]. As for these ferredoxins a departure from the Curie law of the integrated intensity is observed at temperatures higher than 15 K and there is an overall relaxation broadening of the line above 20 K. The absolute intensity measurements give an average of 1 ($\pm$0.1) 3Fe cluster per monomer for Fd(a), and 0.1 ($\pm$0.03) for Fd(an) before this protein was concentrated for the redox titration experiments. After concentration to 0.18 mM on a DEAE cellulose column, the amount of 3Fe clusters in Fd(an) was found equal to 0.2 ($\pm$0.02). These observations indicate that the existence of the 3Fe clusters is strongly related to the presence of oxygen in the purification procedure, so that most of these clusters result very likely from an oxidative conversion of |4Fe-4S| clusters present in the native form of the protein.

When the samples are fully reduced with a large excess of dithionite in solution at pH 7.6, the 3Fe signal disappears completely and is replaced by a spectrum which is typical of two interacting |4Fe-4S| clusters [12] (Fig. 1d). The intensity of this signal was measured for Fd(an) and Fd(a) and corresponds in both cases to 2 ($\pm$0.2) |4Fe-4S| clusters per monomer. This indicates that the 3Fe clusters have been converted into the |4Fe-4S| form. The accuracy of the absolute intensity measurements is not sufficient to decide whether the iron atoms, which are involved in the formation of the |4Fe-4S| clusters, come from a cannibalization process of the 3Fe clusters as in Aconitase [17] or from adventitious iron.

Potentiometric titration

The results of the EPR redox titration experi-

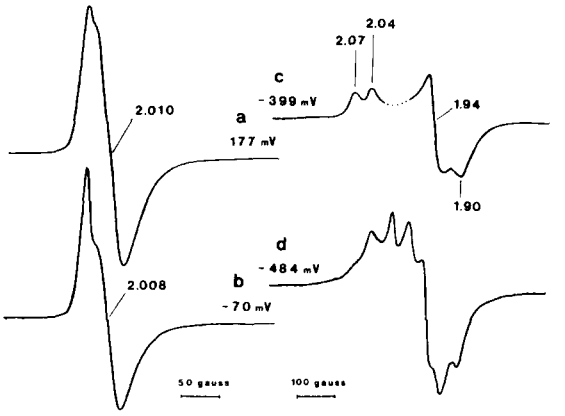

Fig. 1. Typical EPR signals given by *T. commune* Fd(a) at different redox potentials (for experimental conditions, see Materials and Methods). The four spectra a, b, c, d correspond to the samples which give the experimental points noted a, b, c, d on the titration curves in Fig. 2.

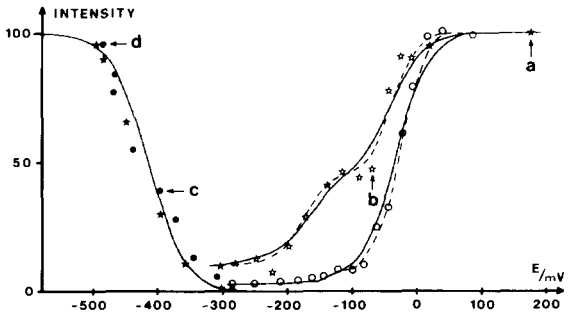

Fig. 2. Redox titration of *T. commune* Fd(a) and Fd(an). The symbols on the titration curves represent the intensity of the EPR signal as a function of the redox potential (relative to the hydrogen electrode). The dots and the stars correspond to Fd(an) and Fd(a), respectively. The experimental sets of data characterized by the different types of symbols were normalized independently and the corresponding intensities are not comparable with each other (for relative intensities, see the text). Above −300 mV, the solid and the dashed lines are the theoretical variations computed for the values of the midpoint potentials given in Table I: ———, the 3Fe-to-4Fe conversion process is neglected; - - - - - -, the conversion process is described by a second order reaction. Below −300 mV the theoretical fit corresponds to an equal contribution of two Nernst curves with midpoint potentials equal to −395 mV and −435 mV, respectively.

ments are given in Fig. 2. The measurements were performed at 15 K and the intensities were obtained by a numerical double integration of the spectra. Two ranges can be distinguished: in the region above −300 mV the signal is given by the 3Fe clusters, whereas only |4Fe-4S| signals are observed in the low-potential range. In the 3Fe region the addition of dithionite in the potentiometric cell induces a fast decrease of the potential which reaches a minimum after about 1 min. This phase corresponds to the reduction of the 3Fe clusters. A slow increase is then observed (half-time of about half an hour) which is probably related to the slow conversion process of some of the 3Fe clusters into a |4Fe-4S| form. This process is much slower than the reduction process so that we will admit that the redox equilibrium of the 3Fe clusters system is satisfied at each time. The presence of a plateau in the experimental titration curve of Fd(a) at about −100 mV (Fig. 2) indicates a large contribution of at least two different 3Fe species (which we named A and B) characterized by different redox properties. The residual contribution below −250 mV is likely due to a third species C with a low redox potential. The presence of different types of 3Fe clusters is also indicated by the evolution of the EPR line shape. In the range +200 mV to −100 mV there is a gradual change from an almost symmetrical shape observed at high potential (Fig. 1a) to the shape shown in Fig. 1b. This can be related to the decrease of the contribution of type A clusters. Below −100 mV the major contribution results from type B clusters and the shape remains unchanged. From the titration curve obtained for Fd(an) it is seen that most of the 3Fe clusters are of type A and this suggests that the formation of type B clusters needs stronger oxidative conditions than for type A.

The decrease of the 3Fe signal induced by dithionite involves at least two processes: one is a fast reduction process and the other a slow conversion of the 3Fe form into a diamagnetic 4Fe structure. In a first approximation one may consider that the conversion process is very slow so that the progressive change in the relative amount of 3Fe and 4Fe clusters is distributed through the whole range of potentials, i.e., from +200 to −500 mV. If we then assume that this amount remains roughly constant in the potential range corresponding to the reduction of one 3Fe species we obtain an estimate of the different midpoint potentials by fitting the data with Nernst curves. The best fit is represented by the solid line in Fig. 2. It was calculated with the midpoint values E_m

(1) and the relative contribution of the different types of 3Fe clusters given in Table I. The values E_m (1) are most likely overestimated. This may be seen by fitting the results with a simple phenomenological model where the conversion process is described by a second order reaction where the conversion rate of the 3Fe clusters is proportional at each time to the dithionite concentration. This model gives a better fit with the experimental data (dashed line in Fig. 2). The best agreement with the results for Fd(an) is obtained for a conversion rate constant of about 500 $min^{-1} \cdot M^{-1}$ and midpoint potential values 40 mV lower than those obtained when the conversion process is ignored (Table I). The same conclusion applies for Fd(a) whose data are also well fitted by using the same rate constant. An estimation of this constant has also been deduced from an independent kinetic experiment performed on Fd(an): a small amount of dithionite was added at two different times in the potentiometric cell containing Fd(an), and a sample was taken and frozen a short time after each addition. The experimental conditions and the data are summarized in Table II where the two EPR samples have been named sample 1 and sample 2. The EPR signal intensity for sample 2 is 30% weaker than that for sample 1, although both samples were taken at the same potential. This difference is attributed to the conversion of 3Fe clusters during the 35 min preceding the new addition of dithionite. If we neglect the number of conversions during the time (2 min) of the reduction of sample 2, we obtain with our simplified model a rate constant value of about 160 $min^{-1} \cdot M^{-1}$, which, considering the crudeness of our assumptions, is consistent with the value deduced from the titration curves.

TABLE II

EXPERIMENTAL CONDITIONS FOR THE KINETIC STUDY OF THE CONVERSION PROCESS In *T. COMMUNE* Fd(an)

Concentration of Fd(an), 77 μM (pH 7.6); concentration of $Na_2S_2O_4$, 4.4 mM. At times 0 and 35 min, dithionite was added just after reading the potential values. The values −57 and −59 mV given for samples 1 and 2, respectively, were measured in the titration cell during the freezing operation of these samples.

Time (min)	Potential in the titration cell (mV)	Addition of $Na_2S_2O_4$ (μl)	EPR signal intensity
0	+88	+3	100%
3	−57		sample 1: 12%
35	+37	+3	
37	−59		sample 2: 8%

According to our interpretation most of the 3Fe clusters have been converted when the potential is lower than −300 mV so that the redox titration experiment should not be perturbed by the conversion process in the |4Fe-4S| region. In this region, the redox properties of Fd(a) and Fd(an) do not show significant differences. A reasonable fit with Nernst curves is obtained by assuming an equal contribution of two |4Fe-4S| clusters characterized by slightly different potentials −395 mV (±10 mV) and −435 mV (±10 mV). These values are similar to those found in bacterial 2|4Fe-4S| ferredoxins [13]. At the beginning of the reduction of

TABLE I

MIDPOINT POTENTIALS E_m DEDUCED FROM THE REDOX TITRATION EXPERIMENTS

Two different approximations have been used: (1) the conversion process is neglected; (2) the conversion process is described by a second-order process where the conversion rate is proportional to the dithionite concentration. In this case it was also assumed for Fd(a) that only the reduced 3Fe clusters could be converted. The common rate constant for Fd(a) and Fd(an) is $k = 500\ min^{-1} \cdot M^{-1}$.

3Fe cluster type	E_m (mV) Fd(a)		3Fe cluster type	E_m (mV) Fd(an)	
	(1)	(2)		(1)	(2)
A (55%)	−40	−80	A (95%)	−30	−70
B (33%)	−170	−310	B (2%)	−170	< −170
C (12%)	< −300	< −300	C (3%)	< −300	< −300

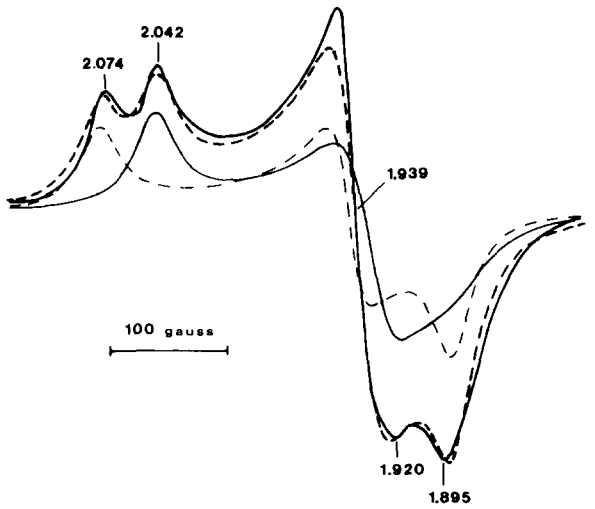

Fig. 3. Numerical simulation of the EPR signal observed at −399 mV (Fig. 1c). The calculated spectrum is represented by the heavy dashed line. It is the superposition of two simulated rhombic spectra of equal intensities and characterized by g_x = 2.075, g_y = 1.94, g_z = 1.89 (- - - - -) and g_x = 2.042, g_y = 1.93, g_z = 1.89 (———). Each of these spectra were obtained by calculating the convolution product between the densities of the packet lines and a lorentzian packet line-shape with a field-dependent width.

the |4Fe-4S| clusters, only one cluster per monomer is reduced and we should observe a typical |4Fe-4S| signal as in the case of bacterial 2|4Fe-4S| ferredoxins [12]. In fact the spectra which are observed in this region for Fd(a) and Fd(an) appear as the superposition of two |4Fe-4S| signals with different g_z values (Fig. 1c). To obtain the main characteristics of these signals we performed a simulation, based on a convolution procedure, of the spectrum given by the Fd(a) sample at −399 mV (Fig. 3). This spectrum is reasonably fitted by assuming an equal contribution of a usual rhombic |4Fe-4S| signal characterized by $g_z \approx 2.07$ and a more axial-looking signal characterized by $g_z \approx 2.04$. It is interesting to note that spectra with similar shapes and characterized by a $g_z \approx 2.04$ component have been reported in two cases: (i) |4Fe-4S| clusters in sites which were initially 3Fe sites [1,3,4,14]; (ii) 4Fe-4S clusters in systems where the constraint on the clusters is weak: partially unfolded proteins [15] or synthetic analogs [16]. This suggests that the 2.04 signal which is observed here could be associated to a weakly constrained site favorable to an easy accommodation of structurally different clusters.

TABLE III

4Fe-TO-3Fe CONVERSIONS IN *T. COMMUNE* FERREDOXIN

The samples are those which were taken at −450 mV for Fd(a) and −372 mV for Fd(an) in the redox titration experiments (Fig. 2). They contain 8-fold excess and 6-fold excess of dithionite, respectively. They were let to thaw and reoxidize by air at room temperature. The number of 3Fe clusters is deduced from absolute intensity measurements of the EPR signals.

Time of reoxidation by air (h)	3Fe clusters per monomer for Fd(a)	3Fe clusters per monomer for Fd(an)
0	0	0
3	0.08	0.07
4	0.21	0.08
5	0.35	0.11
17	0.27	0.30

4Fe-to-3Fe conversions

At the end of the redox titrations, two samples were let to thaw and reoxidize by air at room temperature. They were a Fd(a) sample which had been taken at −450 mV and contains an 8-fold excess of dithionite and a Fd(an) sample which had been taken at −372 mV and contains a 6-fold excess of dithionite. The buffer concentration was high enough (see Materials and Methods) to avoid damaging effects on the cluster structure which would result from a decrease of the pH when oxygen reacts with dithionite. We observed for both samples a slow reappearance of the 3Fe signals. The results are given in Table III. From observations which were done during the titration experiments we know that when a reduced ferredoxin solution is allowed to reoxidize by air, less than an hour is necessary for its potential to return from its lowest value (approx. −480 mV with a 10-fold excess of dithionite) to a positive value at which all the 3Fe clusters are oxidized. This means that the relative intensities of the EPR signals given in Table III are directly proportional to the number of 3Fe clusters obtained by conversion of the 4Fe clusters. Although slow for both samples, the 4Fe-to-3Fe conversion kinetic appears faster for Fd(a) at least in the first hours of the process, thus indicating that the fully reduced forms of Fd(a) and Fd(an) are not identical. This suggests that the interconversion processes take place in

particular conversion sites which are irreversibly formed when oxygen is present during the purification. As mentioned in Ref. 8 and also reported for activated Aconitase [17], a very long exposure to air gives rise to a progressive destruction of the clusters which can explain a limitation of the increase of the 3Fe signals or even a decrease as observed for Fd(a).

Conclusion

The amount of 3Fe clusters in the ferredoxins from *T. commune* is largely dependent upon the presence of oxygen in the purification procedure. This strongly suggests that most of them result from an oxidative conversion of native |4Fe-4S| clusters. The EPR redox titration experiments indicate that at least three types of 3Fe clusters can be accommodated by the protein. They differ by their midpoint potentials: approx. −80 mV, approx. −210 mV and less than −300 mV. These values are within the range of the potentials measured in the other 3Fe proteins (from 0 mV for Aconitase [18] to −420 mV for *A. vinelandii* Fd I [19]). Their EPR spectra and the temperature dependence of the shape and the intensity exhibit the same main characteristics as those reported for *D. gigas* Fd II and *A. vinelandii* Fd I so that all these 3Fe clusters cannot present major structural differences. An easy conversion of these clusters into |4Fe-4S| clusters is observed when dithionite is added at usual pH values, with an half-time longer than 1 h. It is suggested that the site of the protein which is implicated in this process could be related to the unusual $g = 2.04$ |4Fe-4S| signal which is seen in the range −300 mV−−400 mV. A slow 4Fe-to-3Fe conversion process is observed when the fully reduced ferredoxin is reoxidized by air. Below −300 mV most of the clusters are of the |4Fe-4S| type. In the fully reduced state the two clusters in the monomer are magnetically coupled and the close similarity of the EPR spectrum with those observed for other bacterial 2|4Fe-4S| ferredoxins indicates an intercluster distance of about 1.2 nm [20]. The redox midpoint potentials which are about −400 mV are also compatible to those observed in these ferredoxins.

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References

1 Kennedy, M.C., Kent, T.A., Emptage, M., Merkle, H., Beinert, H. and Münck, E. (1984) J. Biol. Chem. 259, 14463–14471
2 Moura, J.J.G., Moura, I., Kent, T.A., Lipscomb, J.D., Huynh, B.H., Le Gall, J., Xavier, A.V. and Münck, E. (1982) J. Biol. Chem. 257, 6259–6267
3 Morgan, T.V., Stephens, P.J., Burgess, B.K. and Stout, C.D. (1984) FEBS Lett. 167, 137–141
4 George, S.J., Richards, A.J.M., Thomson, A.J. and Yates, M.G. (1984) Biochem. J. 224, 247–251
5 Nagayama, K., Imai, T., Ohmori, D. and Oshima, T. (1984) FEBS Lett. 169, 79–84
6 Johnson, M.K., Thomson, A.J., Richards, A.J.M., Peterson, J., Robinson, A.E., Ramsay, R.R. and Singer, T.P. (1984) J. Biol. Chem. 259, 2274–2282
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 79, 1096–1100
8 Papavassiliou, P. and Hatchikian, E.C. (1985) Biochim. Biophys. Acta 810, 1–11
9 Gayda, J.P., Bertrand, P., Theodule, F.-X. and Moura, J.J.G. (1982) J. Chem. Phys. 77, 3387–3391
10 Gayda, J.P., Bertrand, P., Guigliarelli, B. and Meyer, J. (1983) J. Chem. Phys. 79, 5732–5733
11 Bertrand, P., Guigliarelli, B., Meyer, J. and Gayda, J.P. (1984) Biochimie 66, 77–79
12 Mathews, R., Charlton, S., Sands, R.H. and Palmer, G. (1974) J. Biol. Chem. 249, 4326–4328
13 Stombaugh, N.A., Sundquist, J.E., Burris, R.H. and Orme-Johson, W.H. (1976) Biochemistry 15, 2633, 2639
14 Johnson, M.K., Spiro, T.G. and Mortenson, L.E. (1982) J. Biol. Chem. 257, 2447–2452
15 Cammack, R. (1975) Biochem. Soc. Trans. 3, 482–488
16 Laskowski, E.J., Reynolds, J.G., Frankel, R.B., Foner, S., Papaefthymiou, G.C. and Holm, R.H. (1979) J. Am. Chem. Soc. 101, 6562–6570
17 Kennedy, M.C., Emptage, M.H., Dreyer, J.L. and Beinert, H. (1983) J. Biol. Chem. 258, 11098–11105
18 Beinert, H. and Thomson, A.J. (1983) Arch. Biochem. Biophys. 222, 333–361
19 Sweeney, W.V. and Rabinowitz, J.C. (1975) J. Biol. Chem. 250, 7842–7847
20 Adman, E.T., Sieker, L.C. and Jensen, L.H. (1973) J. Biol. Chem. 248, 3987–3996