Oxidation-Reduction Properties of the Two Fe₄S₄ Clusters in Clostridium pasteurianum Ferredoxin*

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The ferredoxin from Clostridium pasteurianum contains two Fe₄S₄ clusters. In this paper we determine their oxidation-reduction midpoint potentials; we find them to be essentially identical (within 10 mV) and to have pH-independent \( E_m \) values of \(-412 \pm 11 \text{ mV}\) from pH 6.3 to 10.0.

Bacterial ferredoxin was first isolated from the anaerobic N₂-fixing bacterium Clostridium pasteurianum in 1962 and found to be an essential electron carrier in several electron transfer processes (1, 2). Since then such proteins have been found in a variety of microorganisms, shown to contain Fe₄S₄ clusters, and have been the subject of extensive research (Ref. 3 and references therein). Because of their vital role as electron mediators in and between various metabolic processes, the oxidation-reduction properties of bacterial ferredoxins have been studied in some detail (4–10), but the sum of the data is confused. Most of the experiments have involved titrations of the optical absorption, which unfortunately is broad and of low extinction coefficient. Furthermore, many of the “titrations” in fact reflect only a single “equilibration” with the \( \text{H}_2/2\text{H}^+ \) couple via hydrogenase. Only one paper has specifically addressed the question of whether the two Fe₄S₄ clusters are identical in their oxidation-reduction properties, and that only at a single pH (11).

In this paper we address the oxidation-reduction properties of the two Fe₄S₄ clusters of C. pasteurianum ferredoxin using redox potentiometry and electron spin resonance spectroscopy. This ferredoxin has proven to be typical of the class, a small protein (\( M_r \), about 6000) containing two Fe₄S₄ clusters with low oxidation-reduction potentials (2, 3). The two Fe₄S₄ clusters have spin = \( \frac{1}{2} \) in the reduced state and are spin coupled to yield a complex esr spectrum (12). This has allowed us to quantitate the fraction of molecules with none, one, or two reduced Fe₄S₄ clusters at various redox potentials and thereby determine the oxidation-reduction midpoint potential \( (E_m) \) of each cluster independently.

We find the two clusters to have essentially identical \( E_m \) values \((-412 \pm 11 \text{ mV})\), which are independent of pH over the range pH 6.3–10.

**MATERIALS AND METHODS**

C. pasteurianum W5 was grown under N₂-fixing conditions as described previously (13). The ferredoxin was purified, essentially as described by Rabinowitz (14), except that the final crystallization step was omitted. The protein had \( A_{280}/A_{260} = 0.79 \). Redox potentiometry followed the methods described by Dutton (15), using 40 \( \mu \text{M} \) methyl and benzyl viologens \( (E_m = -430 \text{ and } -350 \text{ mV}, \text{respectively}) \)

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*The abbreviations used are: mT, millitesla; Mes, 2-(N-morpholino)ethanesulfonic acid; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
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The Nernst equation, as it is used in redox chemistry (see Refs. 18 and 19), may be written as

\[ E_{h} = E_{m} + \frac{RT}{nF} \ln \left( \frac{[\text{oxidized}]}{[\text{reduced}]} \right) \]

where \( E_{h} \) is the ambient redox potential, \( E_{m} \) is the oxidation-reduction midpoint potential of the couple under study, \( R, T, \) and \( F \) have their usual meanings, \( n \) is the number of electrons involved in the redox process, and [oxidized] and [reduced] are the concentrations of the oxidized and reduced species, respectively.

At room temperature and pressure, this may be simplified as follows.

\[ E_{h} = E_{m} + \frac{RT}{nF} \ln \left( \frac{[\text{oxidized}]}{[\text{reduced}]} \right) \]

\[ \text{Let one cluster in the ferredoxin be named } A, \text{ the other B, with } E_{mA} \text{ and } E_{mB} \text{, respectively, and both be } n = 1 \text{ Nernstian species. The fraction of A reduced at any } E_{h} \text{ may then be calculated as} \]

\[ [R_{A}] = \left[ 10^{\frac{E_{h} - E_{mA}}{0.059}} + 1 \right]^{-1} \]

while the fraction of B reduced at any \( E_{h} \) is

\[ [R_{B}] = \left[ 10^{\frac{E_{h} - E_{mB}}{0.059}} + 1 \right]^{-1} \]

The fraction of protein that has both clusters reduced will then be \( R_{A} \cdot R_{B} \), while the fraction that has neither reduced will be \( (1 - R_{A}) \cdot (1 - R_{B}) \). The fraction that has only one cluster reduced will then be \( 1 - [R_{A} \cdot R_{B}] - [(1 - R_{A}) \cdot (1 - R_{B})] \). Such behavior is described for a range of \( E_{mA} \) and \( E_{mB} \) values (expressed as their difference, \( E_{mA} - E_{mB} \)) in Fig. 4. Note that this behavior, where two independent centers each accept a single electron, is quite distinct from that where a single redox species accepts two electrons, as for example, a quinone (see Fig. 5 of Ref. 20).

The data reported here are from esr experiments. This spectroscopy is an excellent comparative technique, but absolute quantitations of mixtures of spectra as complicated as those of Figs. 1 and 2 are difficult. For this reason, Fig. 4 concentrates on those features of esr redox titrations that are most sensitive to the difference in midpoints of two redox species such as those found in \textit{C. pasteurianum} ferredoxin. The upper portion of the figure shows a typical calculation, in this case where \( E_{mA} = E_{mB} = 0 \text{ mV} \). The fraction of the protein that has a single reduced cluster is maximal, at 0.5, at
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FIG. 4. The behavior of two independent \( n = 1 \) couples as a function of the difference between their \( E_m \) values. This figure is discussed in the text. The upper portion is a simulation of the fractions of the protein that are singly reduced (\( F^- \), the hump) or doubly reduced (\( F'' \), the curve that asymptotically approaches 1) when both \( E_{mA} \) and \( E_{mB} \) are 0 mV. Three readily measured parameters, \( A \), \( B \), and \( C \), are shown. The lower portion of the figure shows how these three parameters vary as the difference between \( E_{mA} \) and \( E_{mB} \) is varied.

0 mV, while the fraction of the protein that has two reduced clusters rises with an apparent \( "E_{m}" \) at \(-22 \text{ mV} \). Of course this latter value is not the \( E_m \) of either cluster, but it is a value readily measured in redox titrations such as those in Fig. 3. Two other features are readily obtained in such experiments: the \( E_m \) of maximal singly reduced protein, and hence the difference between this point and the apparent \( E_m \) of the doubly reduced form (\( A \) in Fig. 4), and the "width at half-height" of the titration of the singly reduced protein (\( B \) in Fig. 4). Also readily calculated, although difficult to quantitate in absolute terms in these experiments, is the fraction of protein containing only a singly reduced cluster (\( C \) in Fig. 4). The three parameters discussed above turn out to be sensitive indicators of the difference in \( E_m \) between the two clusters, as shown in the lower part of Fig. 4. Note that these calculations have assumed that both clusters have identical esr properties, so that curve \( C \) is symmetric about 0 mV. Similar calculations can readily model alternative possibilities, such as the possibility that only one of the clusters is detectable in the singly reduced protein.

The data of Fig. 3, together with those to be discussed in Figs. 5 and 6, are all fit very well by assuming that the two clusters of \( C. \) pasteurianum ferredoxin have identical \( E_m \) values and that the maximal difference between the \( E_m \) values, if there is one, must be less than 10 mV.

Fig. 5 shows that there is no significant pH dependence of the \( E_m \) of the clostridial ferredoxin clusters. Neither is there any significant dependence on the concentration of the ferredoxin (Fig. 5). There is a slight dependence on the ionic strength (Fig. 6), with the \( E_m \) increasing with the ionic strength. The data are tabulated in Table I.

DISCUSSION

The data reported in this paper show that the two clusters of the ferredoxin from \( C. \) pasteurianum have essentially identical oxidation-reduction properties (within 10 mV) and that these do not vary significantly as a function of pH. As an appropriate corollary of the pH independence, the \( E_m \) is

FIG. 5. Redox titrations of ferredoxin as a function of its concentration. Ferredoxin at the indicated concentration was titrated as in Fig. 1, except that the buffer was 50 mM sodium pyrophosphate, pH 8.9. Symbols as in Fig. 3.

FIG. 6. Redox titrations of ferredoxin as a function of ionic strength. 228 \( \mu \text{M} \) ferredoxin was titrated as in Fig. 1, except that the buffer was 50 mM glycine, pH 9.4, with and without 1 M KCl. Symbols as in Fig. 3.
slightly dependent on the ionic strength, with the protein becoming easier to reduce as the ionic strength increases.

The determined $E_m$ of *C. pasteurianum* ferredoxin ($-412 \pm 11$ mV) is in reasonable agreement with the values of others (4–10), although our conclusions are somewhat different from those of Magliozzo et al. (10). They found that the $E_m$ varied from −375 mV at pH 6.5 to −438 mV at pH 8.8. These values are not very different from ours (mean of their data = −401 ± 24 mV, mean of ours = −412 ± 11 mV), but ours show no convincing pH dependence. There are several differences in the experimental approaches used in the two laboratories. We have used equilibrium redox potentiometry and esr spectroscopy; Magliozzo et al. (10) used the method of Lode et al. (7), which involves equilibration with the hydrogen electrode via hydrogenase, and measuring the reduction optically. One problem with such a technique is that essentially full reduction (>99%) of the protein would only be expected when its $E_m$ is more than 118 mV positive of that of the hydrogen electrode. This was not achieved in the pH range they used, so estimates of the fully reduced spectrum may have been significantly underestimated. Only at pH values above pH 8.2 do the data of Magliozzo et al. (Fig. 1 in Ref. 10) suggest that they reduced the ferredoxin by more than 90%, and their data set only includes three pH values in this region. In contrast, the approach to fitting the data obtained here, outlined in Fig. 4, allows reasonable extrapolations even at pH values where the protein can be only partially reduced at equilibrium. Nevertheless, Magliozzo et al. (10) provided additional evidence for the pH dependence of the $E_m$ by measuring proton binding during reduction, which although small, seemed to be appropriate for the pH dependence measured in their equilibrium experiments. We are at a loss to explain this part of their data.

Our finding that the two clusters have essentially identical $E_m$ values under all the conditions of pH, concentration, and ionic strength measured here is in reasonable agreement with the finding of Packer et al. (11), who used nmr to estimate the potentials of the two clusters. They concluded that the clusters differed by 10 ± 5 mV in *C. pasteurianum* ferredoxin and by <10 mV in *C. acetobutylicum* ferredoxin. They only measured these values at pH 7.0 (actually in D2O at pH 7.4) so again were unable to fully reduce the ferredoxins at equilibrium.

Our findings on the oxidation-reduction properties of the two Fe3S4 clusters of ferredoxin now need to be extended to other systems where similar esr spectra suggest a pair of interacting clusters, such as in the 12-iron bidirectional hydrogenase of *C. pasteurianum* (21) and nitrate reductase of *Escherichia coli*, which contains three or four ferredoxin Fe3S4 clusters (22). Is the equivalence of electrochemical properties a general feature of such systems?

**REFERENCES**


