High and Low Reduction Potential 4Fe-4S* Clusters in Azotobacter vinelandii (4Fe-4S*)₂ Ferredoxin I

INFLUENCE OF THE POLYPEPTIDE ON THE REDUCTION POTENTIALS*

(Received for publication, April 14, 1975)

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Azotobacter vinelandii (4Fe-4S*)₂ ferredoxin I (Fd I) is an electron transfer protein with $M_r = 14,500$ and $E_0 = -420$ mv. It exhibits an EPR signal of $g = 2.01$ in its isolated form. This resonance is almost identical with the signal that originates from a "super-oxidized" state of the 4Fe-4S* cluster of potassium ferricyanide-treated Clostridium ferredoxin. A cluster that exhibits this EPR signal at $g = 2.01$ is in the same formal oxidation state as the cluster in oxidized Chromatium High-Potential-Iron-Protein (HiPIP). On photoreduction of Fd I with spinach chloroplast fragments, the resonance at $g = 2.01$ vanishes and no EPR signal is observed. This EPR behavior is analogous to that of reduced HiPIP, which also fails to exhibit an EPR spectrum. These characteristics suggest that a cluster in A. vinelandii Fd I functions between the same pair of states on reduction as does the cluster in HiPIP, but with a midpoint reduction potential of $-420$ mv in contrast to the value of $+350$ mv characteristic of HiPIP. Quantitative EPR and stoichiometry studies showed that only one 4Fe-4S* cluster in this (4Fe-4S*)₂ ferredoxin is reduced. Oxidation of Fd I with potassium ferricyanide results in the uptake of 1 electron/mol as determined by quantitative EPR spectroscopy. This indicates that a cluster in Fd I that shows no electron paramagnetic resonance in the isolated form of the protein accepts an electron on oxidation, as indicated by the EPR spectrum, and becomes paramagnetic. The EPR behavior of this oxidizable cluster indicates that it also functions between the same pair of oxidation states as does the Fe-S cluster in HiPIP. The midpoint reduction potential of this cluster is approximately $+340$ mv. A. vinelandii Fd I is the first example of an iron-sulfur protein which contains both a high potential cluster ($+340$ mv) and a low potential cluster ($-420$ mv). Both Fe-S clusters appear to function between the same pair of oxidation states as the single Fe-S cluster in Chromatium HiPIP, although the midpoint reduction potentials of the two clusters are $\sim 760$ mv different.

Azotobacter vinelandii, a nitrogen-fixing aerobic bacterium, has been shown to contain a number of iron sulfur proteins (1). In addition to the nitrogenase, which is an iron-sulfur protein complex, this organism contains two 2Fe-2S* proteins (called Azotobacter iron-sulfur proteins I and II (2)) and two proteins containing one or more 4Fe-4S* clusters (3-5). An 8-Fe protein (two 4Fe-4S* clusters) previously referred to as either ferredoxin I (Fd I) (5) or iron-sulfur protein III (4), has been obtained in crystalline form with $M_r = 14,500$ and a midpoint reduction potential ($E_{mp}$) of $-420$ mv (5). This A. vinelandii protein will be referred to as A. vinelandii (4Fe-4S*), Fd or Fd I.

The protein can replace spinach ferredoxin in the photoreduction of NADP by spinach chloroplasts and it will couple the reducing power of illuminated spinach chloroplasts to Azotobacter nitrogenase. In addition, Fd I functions with low activity in the clostridial phosphoroclastic reaction (5). Its optical spectrum resembles that of other ferredoxins with $e_{380} = 27,000$ M⁻¹ cm⁻¹ and an $A_{400}/A_{280}$ value of 0.58 (5).

Peptococcus aerogenes ferredoxin contains two 4Fe-4S* clusters (6) and functions as an electron transfer protein with $E_0 = -427$ mv (7). Chromatium High-Potential-Iron-Protein (HiPIP), 1 a protein that contains a single 4Fe-4S* cluster (8), has a reduction potential of $+350$ mv (9, 10). X-ray studies of

1 Results described in this paper indicate that separation of iron-sulfur proteins according to their reduction potentials may not be advisable. Although the term High-Potential-Iron-Protein (HiPIP) does convey useful information, we feel that the name Chromatium 4Fe-4S* ferredoxin would be more suitable. However, to avoid possible confusion we will continue to refer to this protein as Chromatium HiPIP until the IUPAC-IUB Commission on Biochemical Nomenclature publishes a revised set of recommendations.
oxidized P. aerogenes ferredoxin (6) and both oxidized and reduced Chromatium HiPIP (11, 12) have shown that the iron-sulfur clusters in both proteins are cube-like structures bound to the polypeptide chain through a cysteinyl sulfur bond to each iron atom. These clusters will be denoted as $[\text{Fe}_x\text{S}^+_y\text{S}_z^+\text{Cys}]^m$. In view of their very different reduction potentials, the similarity in the structure of the iron-sulfur clusters in the two proteins is surprising. It has been proposed that the cluster may exist in three different oxidation states, with clusters in both oxidized ferredoxin and reduced HiPIP in the intermediate oxidation state (11, 13). In this proposal, reduced ferredoxin occupies the lowest oxidation state and oxidized HiPIP, the highest state. This theory is substantiated by the similarity of the optical and magnetic properties of reduced HiPIP and the oxidized ferredoxin (11, 13). In addition, Cammack (14) has been able to further reduce oxidized Chromatium HiPIP with dithionite, and the EPR spectrum exhibited by this "super-reduced" HiPIP is similar to that exhibited by reduced Clostridium ferredoxin. It is stated (11) that the ferredoxin polypeptide stabilizes a pair of oxidation states that are native to each protein and that the potential of each protein reflects the pair of oxidation states that is stabilized. However, the studies reported here indicate that the potential of the stabilized pair of oxidation states may vary significantly (>700 mv).

In studies of synthetic analogs of the $[\text{Fe}_x\text{S}^+_y\text{S}_z^+\text{Cys}]^m$ clusters with the general formula $[\text{Fe}_x\text{S}^+_y\text{S}_z^+\text{Cys}]^m$, it has been shown that the cluster is stable in at least four different oxidation states (15). By comparing the physical properties of model compounds with their protein counterparts (13, 15-17) it has been possible to assign over-all oxidation levels to the clusters. According to this scheme, $[\text{Fe}_x\text{S}^+_y\text{S}_z^+\text{Cys}]^m = 1$ is the cluster oxidized HiPIP, $-2$ is the charge on the clusters in reduced HiPIP and oxidized ferredoxin, and $-3$ is the charge on the clusters in reduced ferredoxin. The use of net oxidation numbers reflects a lack of knowledge of the details of the electronic structure of the cluster. The EPR behavior shown by the 8Fe-8S* ferredoxin (Fd I) from A. vinelandii appears to differ from other 8Fe-8S* ferredoxins. Shethna (4) has reported an EPR spectrum of the dithionite-treated Fd I that is similar to that of the $g = 2.01$ resonance arising from super-oxidized Clostridium Fd (18). Experiments in this laboratory indicated that the $g = 2.01$ resonance is exhibited by A. vinelandii Fd I in its isolated form. In fact, this resonance is found to be of much greater intensity in the isolated protein than is the resonance exhibited by the dithionite-treated protein or by the isolated form of Clostridium Fd. This suggested that the isolated form of A. vinelandii Fd I may be equivalent to super-oxidized Clostridium Fd. For this reason a study of the EPR and oxidation-reduction properties of A. vinelandii Fd I was undertaken.

**MATERIALS AND METHODS**

Azotobacter vinelandii was grown and Fd I was extracted according to previously published procedures (5).

Spinach chloroplast fragments without oxygen-evolving capacity (19) were prepared according to the method of Yoch and Tagawa (20) and were used for the photoreduction of ferredoxin. Reductions of ferredoxins using hydrogen/hydrogenase were performed according to the method of Tagawa and Arnon (21).

EPR measurements were performed using a modified (22) X-band JEOL Me-1X spectrometer kindly made available by Dr. Alan J. Bearden.

Quantitative EPR experiments were performed by double integration of the EPR spectrum and comparison to a spin standard. Both copper ethylenediaminetetraacetate and fully reduced Clostridium acidi-urici ferredoxin were used as spin standards. The intensity of the EPR signal at $g = 2.01$ exhibited by A. vinelandii Fd I, observed from 9.8-19.4 K, was found to obey the Curie Law as is required in order to obtain a meaningful number of unpaired electron spins.

**RESULTS AND DISCUSSION**

Reducible Cluster—The EPR spectrum of Azotobacter vinelandii Fd I (Fig. 1) is almost identical with that exhibited by oxidized Clostridium Fd (18, 23, 24). It has an effective $g$ value of 2.012, a line width of 35 G, and the same general lineshape as the nearly isotropic clostridial Fd EPR signal. However, the signal displayed by Azotobacter Fd I is as much as 50 times more intense than the oxidized clostridial Fd signal. An intensity equivalent to 1.0 unpaired electron spin (suggesting the involvement of one cluster) is found in the spectrum of oxidized Clostridium ferredoxin. In oxidized Clostridium Fd this EPR signal has been shown to arise from a small number of 4Fe-4S* clusters in a super-oxidized state (18).

Reduction of Fd I with the hydrogen/hydrogenase system induces both an optical change (Fig. 2) and a reduction in the intensity of the EPR signal at $g = 2.01$. On photoreduction with spinach chloroplast fragments the $g = 2.01$ signal is no longer detectable (Fig. 1). No resonance similar to that exhibited by reduced Clostridium ferredoxins (one $g$ value at 1.94, with an average $g$ value of ~1.96) was observed. It appears therefore, that the oxidized state of Fd I exhibits EPR at $g = 2.01$ while the reduced form exhibits no EPR signal. This EPR behavior is identical with that exhibited by clusters in super-oxidized (g = 2.01) and oxidized (no EPR signal) Clostridium ferredoxin. The oxidation states of the clusters in super-oxidized and oxidized Clostridium ferredoxins have been shown to be the same as those in oxidized and reduced Chromatium HiPIP (18). The implication is that the cluster in Fd I giving rise to this EPR behavior functions between the same pair of oxidation states as HiPIP.

Results of quantitative EPR experiments were used to calculate the reduction potential of Fd I, and these results were
in agreement with the potential determined optically. The reduction potential in millivolts of hydrogen/hydrogenase reduced Fd I, based on the assumption that only 1 electron is transferred at a time, was calculated from:

$$E_{np} = -59 \log \left[ \frac{[4Fe-4S^{+}]_{red}}{[4Fe-4S^{+}]_{ox}} \right] - 59[pH]$$

As has been published previously (5), $E_{np} = -420$ mv when the ratio $[4Fe-4S^{+}]_{red}/[4Fe-4S^{+}]_{ox}$ was determined optically. If this ratio is calculated on the basis of quantitative EPR, a reduction potential of $-450$ mv is obtained. This shows that these EPR states are truly coupled to the reduction of the protein rather than being artifacts resulting from treatment with a reductant. The $30$-mv difference between the optically and EPR-determined potentials may result from a temperature dependence of the potential. Optical spectra were recorded at room temperature while EPR spectra were observed at 10 to 20 K. A similar difference was found between the EPR- and optically determined reduction potentials of spinach ferredoxin (27).

The EPR results (Fig. 1) show the occurrence of an Fe-S* cluster in A. vinelandii Fd I in its isolated state that is analogous to the clusters in ferricyanide-treated Clostridium ferredoxin. This Fd I cluster has a formal valence of minus one that is identical with that of the cluster in oxidized HiPIP. Therefore, on reduction, this Fe-S* cluster in Fd I functions between the same pair of oxidation states as does the cluster of HiPIP. However, the reduction potential of A. vinelandii Fd I is $-420$ mv, as opposed to $+350$ for Chromatium HiPIP. This 770-mv difference constitutes direct evidence of the involvement of the polypeptide in determining the reduction potential of this oxidized-reduced $[Fe,S^{+}_2S^{2-}]_{1/2}/[Fe,S^{+}_2S^{2-}]_{1/2}$ couple.

Because Fd I is an 8Fe-8S* protein, it is assumed to have two 4Fe-4S* clusters, indicated by the designation $[4Fe-4S^{+}]_2$Fd. This view is substantiated by the similarity in the position of the maxima in the optical spectra of A. vinelandii Fd I, Clostridium acidi-urici Fd, and Bacillus polymyxa Fd I, and by their similar molar extinction coefficients per cluster near 400 nm (approximately 4000 m$^{-1}$ cm$^{-1}$ per iron atom) (5, 28, 29). In addition, these three proteins exhibit an EPR resonance at $g = 2.01$, a signal which is not exhibited by any other iron-sulfur protein known to have a different cluster structure.

A. vinelandii ($4Fe\text{-}4S^{+}$)$_2$ Fd I may be expected to accept 2 electrons on reduction because it has two 4Fe-4S* clusters. The isolated form of the protein exhibits an EPR spectrum characteristic of $[Fe,S^{+}_2S^{2-}]_{1/2}$ (oxidized) clusters. Because the reduction potential is so negative ($E_{np} = -420$ mv), both clusters would be expected to be oxidized ($[Fe,S^{+}_2S^{2-}]_{1/2}$) in the isolated protein. Since the minus one state exhibits the $g = 2.01$ signal, the EPR spectrum of the isolated form of Fd I should display an intensity equivalent to 2 unpaired (spin = ½) electrons. However, quantitative EPR experiments show an intensity equivalent to only 1.0 unpaired electron spin (Table I), suggesting that only 1 electron is taken up on reduction (i.e. only one cluster is functioning on reduction). To verify this result, the ferredoxin was photoreduced with heated spinach chloroplasts using ascorbate-reduced 2,6-dichlorophenol indophenol as the source of electrons. The light was then turned off and NADP was added anaerobically resulting in the reoxidation of the ferredoxin and a concomitant reduction of the NADP. By determining optically at 340 and 420 nm the molar ratio of NADP reduced to ferredoxin reoxidized, the number of electrons transferred per mol of ferredoxin could be calculated (21). The results are shown in Table I. The average value of 0.92 electron transferred per Azotobacter Fd I molecule obtained in this manner is in good agreement with the value of 1.0 spin obtained by quantitative EPR (Table I). Results obtained with spinach and clostridial ferredoxins are included in Table I as controls. (Two electrons per mol were expected for clostridial ferredoxin, and the observed number of electrons transferred is within 16% of the expected number. One electron per mol was expected to be transferred by spinach ferredoxin and 1.03 electrons per mol were found.) These data indicate that only one cluster in Fd I is functioning on reduction with hydrogen/
hydrogenase, dithionite, or on photoreduction with spinach chloroplasts.

**Oxidizable Cluster—Addition of potassium ferricyanide (K₃Fe(CN)₆) to A. vinelandii (4Fe-4S*)₂ ferredoxin I causes a change in both the optical spectrum (most apparent above 475 nm, see Fig. 3) and the EPR spectrum (Fig. 4). While the change in the optical spectrum is not large, it is reproducible and reversible. The calculated optical difference spectrum exhibits a maximum between 450 and 425 nm. The low and high field shoulders of the g = 2.01 resonance evident in the EPR spectrum of ferricyanide-treated Fd I may result from spin-spin interaction. No half-field transition indicative of spin-spin interaction was observed in the EPR spectrum, but this may be a result of the expected low transition probability for this resonance. Quantitative EPR of the spectrum of the ferricyanide treated Fd I showed an intensity equivalent to 2.1 unpaired electron spins in the doubly integrated EPR spectrum. This implies that the second cluster in Fd I has given up an electron on oxidation and becomes paramagnetic. This oxidation is reversible, as shown by recovery of 80 to 95% of the optical absorbance at 400 nm (Fig. 3) and a return to the initial EPR spectrum of the protein. To demonstrate the reversibility of the ferricyanide-treated Fd I, it was necessary to reduce the protein with dithionite and then air-reoxidize it to obtain the isolated form of the protein.

Because of the reaction of the Fd with the ferricyanide is slow, and there was some loss of protein upon treatment with ferricyanide, it was only possible to obtain an estimate of the reduction potential. The potential, estimated optically, was found to be +340 ± 50 mv. Care was taken to account for the variable midpoint potential of the ferricyanide/ferrocyanide couple (30). No oxidation of Fd I by p-benzoquinone was observed.

On the basis of these results we believe that the following processes are occurring in A. vinelandii (4Fe-4S*)₂ ferredoxin.

1. On reduction one of the two 4Fe-4S⁺ clusters in the protein accepts an electron:

\[ \text{Fe}_4\text{S}_4^*\text{S}_4\text{S}_4^* \text{F} \text{F}^\text{H} + e^- \rightarrow \text{Fe}_4\text{S}_4^*\text{S}_4\text{S}_4^* \text{F} \text{F}^\text{H}^- \]

and that this reduction involves the same pair of oxidation states (-1 and -2) as in the reduction of HiPIP. This conclusion is supported by a comparison of the optical spectra of these iron-sulfur proteins showing (Fig. 2) that the extent of change on reduction of A. vinelandii Fd I resembles the change...
exhibited by *Chromatium* HiPIP (~1 to -2) rather than that of *B. polymyxa* Fd I (-2 to -3). It is important to remember that the absorbance of both *Chromatium* HiPIP and the isolated form of A. vinelandii Fd I increase on oxidation (Figs. 2 and 3). The assignment of this pair of oxidation states (Table II) is consistent with the observation that the absorbance of both *Chromatium* HiPIP and the isolated form of A. vinelandii Fd I increase on oxidation (Figs. 2 and 3).

### Table II

<table>
<thead>
<tr>
<th>Ferredoxin</th>
<th>Reducible (Fe₅S⁹) cluster</th>
<th>Oxidizable (Fe₅S⁷) cluster</th>
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</thead>
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<td>-1</td>
</tr>
<tr>
<td>As isolated</td>
<td>-1</td>
<td>-2</td>
</tr>
<tr>
<td>Reduced</td>
<td>-2</td>
<td>-2</td>
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Influence of Polypeptide on Reduction Potential—The single iron-sulfur cluster in *Chromatium* HiPIP and both the high and low potential iron-sulfur clusters in A. vinelandii (4Fe-4S)₂ ferredoxin I function between the -1 and -2 oxidation states. However, the potentials of these clusters are +350 mv (9, 10), +340 mv, and -420 mv (5), respectively. Clearly there is significant polypeptide influence on the reduction potential of the [Fe₅S⁹⁺]⁻⁻/[Fe₅S⁷⁺]⁻⁻ couple in these proteins.

There are at least three possible mechanisms by which the polypeptide could exert this influence. One mechanism would require that the cluster have slightly different geometries in its two oxidation states. This has, in fact, been demonstrated for the cluster in *Chromatium* HiPIP by x-ray studies (12). The magnitude and sign of the reduction potential depends on the difference in energy of the two oxidation-reduction states. One might envision the polypeptide restricting the geometry of the cluster in one of the oxidation states to a high energy form, and thereby modifying the reduction potential.

A second possible mechanism of polypeptide influence would be formation of an additional bond between the cluster and the polypeptide in one of the oxidation states. Thus, a bond formed in the -1 oxidation state would lower the energy of this state, leading to a more negative reduction potential.

Finally, it is possible that the two clusters are close enough together to allow strong electronic interaction. The presence of spin-spin interaction suggested by the EPR spectrum of K₃Fe(CN)₆-treated Fd I would be consistent with a small intercluster separation. However, spin-spin interaction has been shown to be present between the two clusters in fully reduced clostridial type ferredoxins (31), and both clusters in these proteins are low potential. Our data do not allow us to choose among these possible mechanisms.

### CONCLUSIONS

A. vinelandii (4Fe-4S)₂ ferredoxin I represents a previously unrecognized type of iron-sulfur protein. The properties of this protein as described here suggest that: (a) classification of iron-sulfur proteins by their reduction potentials may not be sufficiently versatile. The oxidation-reduction properties of Fd I suggest that the iron-sulfur cluster structure is a more fundamental and useful property for classification of these proteins. Thus, 4Fe-4S high-potential-iron-proteins and 4Fe-4S ferredoxins would fall in the same class of protein, rather than being in separate classes, as is currently accepted. (b) The polypeptide can influence the reduction potential of iron-sulfur proteins not only by selecting the functioning oxidation-reduction couple (-1 to -2) or (-2 to -3), but also by exerting a direct influence on the potential of the selected couple. Because the oxidized-reduced [Fe₅S⁹⁺]⁻⁻/[Fe₅S⁷⁺]⁻⁻ couple exhibits a reduction potential of +350 mv in *Chromatium* HiPIP and a -420 mv potential in A. vinelandii Fd I, the polypeptide chain can influence the reduction potential of the (-1 to -2) couple by at least 770 mv, and thus determine the potentials at which these electron transfer proteins function.

### REFERENCES

High and low reduction potential 4Fe-4S clusters in Azotobacter vinelandii (4Fe-4S) 2ferredoxin I. Influence of the polypeptide on the reduction potentials.
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