Origin of the pH Dependence of the Midpoint Reduction Potential in *Clostridium pasteurianum* Ferredoxin: Oxidation State-dependent Hydrogen Ion Association*

(Received for publication, March 2, 1981, and in revised form, December 14, 1981)

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The origin of the dependence of $E_{1/2}$ on pH exhibited by *Clostridium pasteurianum* 2(4Fe-4S) ferredoxin has been investigated. The results show that oxidation state-dependent pK values, which may arise from sites on the iron-sulfur centers, are responsible for the pH effect. Based on a model of two equivalent protonation sites/molecule, values of 7.4 for pK$_{a1}$ and 8.9 for pK$_{a2}$ were obtained. The results of experiments which monitor changes in the hydrogen ion concentration with changes in protein oxidation state are reported. The magnitude of the changes in pH on reduction or reoxidation of the protein are in reasonable agreement with the proposed model. The conformation of *C. pasteurianum* ferredoxin was examined by nmr, epr, and CD spectroscopies to rule out a pH-dependent conformation equilibrium as the origin of the pH effect.

Many iron-sulfur proteins exhibit pH-dependent midpoint reduction potentials and some examples of these are listed in Table I (1–5). Although such dependences have been known for years, the question of the origin of the effect has not been addressed in the literature. It appears reasonable that oxidation state-dependent hydrogen ion binding is a feature of the chemistry of Fe-S centers in general, in view of the following: hydrogenase is an iron-sulfur protein; iron-sulfur proteins may be involved in proton translocation in energy-transducing membranes (5, 6); and the observation that many iron-sulfur proteins exhibit pH-dependent midpoint reduction potentials despite varied polypeptide compositions. To test for the existence of oxidation state-dependent hydrogen ion equilibria, the pH dependence of the midpoint reduction potential of *Clostridium pasteurianum* 2(4Fe-4S) ferredoxin has been examined. This ferredoxin is well characterized, has no amino acid residue with an intrinsic pK between 6 and 9, and exhibits a significant and well defined pH dependence. It is a low molecular weight, clostridial-type ferredoxin with a reduction potential near –400 mV.

**MATERIALS AND METHODS**

*C. pasteurianum* was grown and ferredoxin isolated according to the procedure of Rabinowitz (7).

Reduction potentials were determined using partially purified *C. pasteurianum* hydrogenase, as described by Lode et al. (3). Ferredoxin solutions were prepared from a highly concentrated stock solution by dilution into the buffer of desired pH. All solutions were prepared to be approximately 0.03 mM ferredoxin in 0.1 M Tris-acetate-glycerine-phosphate buffer containing 0.5 mM NaCl. The pH of reoxidized ferredoxin solutions was measured using a Radiometer pH meter, R 25, and this final pH value was used in the calculations. (Small changes in pH occurred upon reduction of certain samples, although the changes were generally not larger than 0.05 pH unit compared to the starting buffer.) The pH of buffers used for 1H-nmr samples was calculated by adding 0.64 to the pH meter reading (5). Optical measurements were made using a Cary 219 UV-visible spectrophotometer.

Proton nmr spectra were recorded on a Bruker 270 MHz spectrometer at the NIH Regional Facility in New Haven, CT. epr spectra were obtained using a Varian V-4500 X-band spectrometer equipped with a Helitran liquid helium transfer system (Air Products). Circular dichroism spectra were recorded on a Jasco J-20 spectropolarimeter.

Electrochemically reduced methyl viologen (Princeton Applied Research, Model 300 Constant Potential Coulometry System) was used in experiments which determined the number of micromoles of hydrogen ion bound or released. The pH measurements were made using a Radiometer PHM 26 meter equipped with a combination microelectrode in an anaerobic cell which permitted additions through a serum cap. Since aerobic oxidation of ferredoxin or methyl viologen leads to an increase in pH, care was taken to ensure strict anaerobic conditions. All solutions were prepared in 1.0 mM Tris-Cl buffer containing 0.50 mM NaCl. For the reduction experiments, the pH of the methyl viologen solution was adjusted to match that of the protein solution, and for the reoxidation experiments, the pH of the ferredoxin solution was adjusted to match that of the methyl viologen/ferredoxin solution.

**RESULTS AND DISCUSSION**

The pH dependence of the midpoint reduction potential, $E_{1/2}$, for *C. pasteurianum* ferredoxin was determined from pH 6.2 to pH 8.9. At pH values lower than 7.4 $E_{1/2}$ exhibits a dependence of −16 mV/pH unit and at pH values greater than 7.4, a dependence of −30 mV/pH unit is observed. These results are consistent with a number of previously reported results (1, 3, 9, 10, 11).

Two mechanisms by which the observed midpoint reduction potential can exhibit a pH dependence are oxidation-reduction equilibria involving hydrogen ion binding, and pH-dependent protein conformation changes.

To test the possibility that oxidation state-dependent hydrogen ion binding occurs, the experimental data were compared to a calculated pH dependence curve. A pH dependence can be incorporated into the Nernst equation using the model

$$E_{1/2} = E^\circ + \frac{RT}{nF} \ln \left( \frac{[H^+]^{pK_{a1}}}{[H^+]^{pK_{a2}}} \right)$$

The data in Ref. 9 were recalculated using a value of $n = 1$ in the Nernst equation. The data in Ref. 10 were recalculated using 0.435 as the ratio of extinction coefficients of reduced to oxidized ferredoxin at 425 nm.
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of two equivalent sites/protein molecule:

\[
(4\text{Fe}-4\text{S})_0 + e^- + H^+ \rightarrow (4\text{Fe}-4\text{S})_1 + H^+ \\
K_{\text{ox}} E_{1/2}^\text{p} + K_{\text{red}} E_{1/2}^\text{p} \\
(4\text{Fe}-4\text{S})_0 \rightarrow H^+ + e^- (4\text{Fe}-4\text{S})_1 - H^+ 
\]

The diagram above is only schematic. It is not required that oxidation state-dependent proton binding (for example, a single titrable site with more widely separated pK values) also yield reasonable fits to the experimental data. The model of two equivalent sites/protein molecule was chosen for the sake of ease of calculation and does yield an excellent fit with values of 7.4 ± 0.1 for \( pK_{\text{ox}} \) and 8.9 ± 0.2 for \( pK_{\text{red}} \), \( E_{1/2} = -371 \text{ mV} \).

To test further for the possibility of oxidation state-dependent hydrogen ion binding, experiments were designed to determine the change in hydrogen ion concentration in a solution of ferredoxin upon a change in protein oxidation state. In the first type of experiment, ferredoxin was incubated anaerobically and the pH was determined. Electrolytically reduced methyl viologen was added in excess to reduce the ferredoxin and the pH of the solution was measured again.

A reproducible difference was observed between the solution of oxidized and reduced ferredoxin (Table II) indicating the presence of sites of oxidation state-dependent proton binding. Using a titration curve determined for an identical sample of protein, the hydrogen ion binding to the protein on reduction was quantitated from the observed pH difference (Table II). These experimentally obtained results were compared to a calculated difference in bound hydrogen ion based on the model of two equivalent sites with \( pK_{\text{ox}} = 7.4, pK_{\text{red}} = 8.9 \).

**Table I**

The pH dependence of the midpoint reduction potential of several iron-sulfur proteins

| Cluster type | Source | Estimated pK dependence | pH range |
|--------------|--------|-------------------------|----------|
| 2Fe-2S       | Spinach | -4                      | 7.0-8.2  |
|              | Pseudomonas putida | -30                   | 7.5-    |
|              | Parsley | -7                      | 6.0-9.0  |
| 2Fe-2Se      | Parsley | -14                     | 6.5-8.0  |
| 4Fe-4S       | Peptococcus aergenesis | -8                 | 6.9-7.6  |
|              | Clostridium pasteurianum | -16             | 6.6-7.5  |
|              |    | -12                     | 6.7-7.7  |
|              | Clostridium acidi urici | -13                | 6.5-8.2  |
|              |    | -2                      | 7.0-7.9  |
|              | Clostridium tartarivorum | -3                | 7.0-7.9  |
|              | Bacillus polymyxa (I) | -11                | 6.6-7.7  |
|              | Bacillus polymyxa (II) | -24               | 6.9-7.7  |
|              | Chromatium vinosum | -11                 | 8.1-8.9  |
|              | Chromatium vinosum (HiPIP) | 0              | 7.0-11.0 |
| 2Fe-2S       | Ox heart mitochondria, center N-1a | -60             | 6.2-8.6  |
| 4Fe-4S       | Ox heart mitochondria, center N-2 | -60             | 6.8-8.6  |

**Table II**

Change in hydrogen ion concentration on reduction of C. pasteurianum ferredoxin

An 800-pl sample of 0.53 mM C. pasteurianum ferredoxin in 1 mM Tris-HCl buffer/500 mM NaCl, at the indicated pH value, was made anaerobic by continuous flushing of a stirred sample with oxygen-free nitrogen in a cell which also contained a combination pH electrode. The sample was reduced by addition of 100 pl of 50 mM reduced methyl viologen. The number of micromoles of hydrogen bound was determined from the observed pH values by comparison with titration curves obtained for solutions of the buffer alone (Experiments 1, 2, 3, and 3) and the buffered protein solution (Experiments 4, 5, and 6). Because of the relatively large effect of addition of methyl viologen to a sample of buffer containing no protein, a correction was made to include this factor in a calculation of the total number of micromoles of \( H^+ \) bound on reduction, as indicated in the table. A calculation of an expected number of micromoles of hydrogen ion bound on reduction was made based on a model of two equivalent sites of hydrogen ion binding/ferredoxin molecule, with \( pK_{\text{ox}} = 7.4, pK_{\text{red}} = 8.9 \).

| Experiment | Ferredoxin | pH, initial | pH, after addition of methyl viologen | \( H^+ \) bound | \( H^+ \) bound + 0.101 | Predicted by theory | Theoretical prediction |
|------------|------------|------------|-------------------------------------|----------------|------------------------|---------------------|------------------------|
|            |            |            |                                     |                |                        |                     |                        |
| 1          |            |            | 8.07                                | 7.83           | -0.117                 |                     |                        |
| 2          |            |            | 8.11                                | 7.95           | -0.081                 |                     |                        |
| 3          |            |            | 8.03                                | 7.82           | -0.099                 |                     |                        |
| Average \( \mu \text{mole} \) \( H^+ \) bound | 4          | 0.42               | 7.87                                | 8.32           | 0.284                  | 0.385               | 0.445                  | 87                      |
|            | 5          | 0.42               | 7.72                                | 8.36           | 0.389                  | 0.490               | 0.389                  | 126                     |
|            | 6          | 0.42               | 7.67                                | 8.10           | 0.241                  | 0.342               | 0.440                  | 78                      |
As shown in Table II, the agreement is very good between the experimental and predicted decrease in hydrogen ion concentration on reduction of ferredoxin.

A second type of experiment determined the amount of hydrogen ion released upon oxidation of reduced ferredoxin. Ferredoxin was anaerobically reduced with methyl viologen, the pH was determined, and the sample was reoxidized using a minimum amount of potassium ferrocyanide. Once again, a difference in pH between reduced and oxidized ferredoxin was observed, and this difference was used to determine the amount of hydrogen released by comparison to a titration curve. As can be seen in Table III, the agreement between experiment and theory is excellent. Control experiments indicate that the known destructive side reactions of ferricyanide with ferredoxin do not alter the pH in the period of time required for the reoxidation experiment (2-5 min). The average quantitative result for the reduction and oxidation experiments is 95% of the predicted result.

The quality of the fit of the experimental data to the calculated curve in Fig. 1, the experimental demonstration of a change in hydrogen ion concentration upon change of ferredoxin oxidation state, and the quantitative agreement between this change and that theoretically predicted, all strongly indicate that the origin of the pH dependence of the midpoint reduction potential is oxidation state-dependent hydrogen ion binding.

To confirm this hypothesis, the alternate possible origin of a pH-dependent $E_{1/2}$, a pH-dependent protein conformation, was examined. In order for a pH-dependent change in conformation to affect the reduction potential without oxidation state-dependent proton association, it must be coupled to some alteration of the 4Fe-4S clusters. In order to detect the presence of a change in protein conformation with pH, nmr, epr, and CD spectra were recorded as a function of pH.

The $^1$H-nmr spectra of oxidized *C. pasteurianum* ferredoxin at pH 6.8 and pH 8.9 appear in Fig. 2. The resonances in the 0-8 ppm region (from 2,2-dimethyl-2-silapentanesulfonic acid) arise from aromatic and aliphatic side chains in the polypeptide and, because of poor resolution, provide no clear insight into the cluster geometry. The relevant resonances appear in the 10-20 ppm region. These resonances arise from the β-cysteinyl protons (13, 14), which appear as single proton resonances shifted downfield due to the paramagnetism of oxidized 4Fe-4S centers at room temperature (13). The position of these resonances depends on contact shift interactions and are thus sensitive to the orientation of the β-carbon to sulfur bonds (15). The resonance positions exhibited by the β-cysteiny1 protons, therefore, provide information concerning the geometry of Fe-S centers and their neighboring atoms. Thus, the $^1$H-nmr spectra are useful in identifying conformation changes at the cluster. As can be seen from Fig. 2, the resonances in the 10-20 ppm region occur at identical field strengths at pH 6.8 and pH 8.9. It therefore can be concluded that no detectable conformational differences exist at the immediate environment of the clusters in oxidized *C. pasteurianum* ferredoxin between these two pH values.

To further examine conformation as a function of pH, and to include the reduced state of ferredoxin, epr spectra were compared at the pH values shown in Fig. 3. Two sets of spectra appear; the first pair (Fig. 3A) are the spectra of partially reduced ferredoxin samples (~15% reduction); the second pair (Fig. 3B) are the spectra of more fully reduced ferredoxin samples (75-90% reduced). Slightly reduced samples of ferredoxin exhibit spectra which arise predominantly from reduced 4Fe-4S centers on one-electron reduced molecules. Fully reduced samples exhibit a more complex spectrum which arises from interaction between two paramagnetic reduced 4Fe-4S centers (16). In either case, the linewidths and $g$ values of the signals are sensitive to the bonding geometry of the 4Fe-4S centers. The spectrum of a slightly reduced sample exhibits a small component of the two-electron reduced spectrum, visible as small peaks centered around 3.3 kG. These features correspond to the two major peaks of the fully reduced ferredoxin spectrum.

The observation that the spectra in Fig. 3 are essentially superimposable at the pH values given, indicates that not only is the geometry at a single cluster conserved (partially reduced samples) but also that the conformations at pH 7 and pH 9 are similar enough to conserve the spin interaction between paramagnetic centers in the same molecule. (The small differences that are visible between the spectra of the two near fully reduced samples may be assigned to slightly different degrees of reduction.)

The epr data presented corroborate the $^1$H-nmr findings, indicating that the conformation of *C. pasteurianum* ferredoxin is pH independent. CD spectra of oxidized ferredoxin were recorded from 300 to 800 nm at pH 6.5 and pH 8.5 and were found to be identical. The CD spectrum of reduced ferredoxin from 300 to 800 nm was also pH-independent from pH 6.5 to 8.3.

![Fig. 2. 270 MHz $^1$H-nmr spectra of oxidized *C. pasteurianum* ferredoxin (22 °C). Samples were prepared on 0.04 M (K,D)$_2$PO$_4$ (K,D)$_2$PO$_4$/0.5 M NaCl containing 0.1% 2,2-dimethyl-2-silapentanesulfonic acid. Ferredoxin concentration, 5 mg/0.14 ml (6 mm).](https://www.jbc.org/Downloaded_from/www.jbc.org by guest on March 24, 2020)
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The results presented in this paper demonstrate that the pH dependence of the midpoint reduction potential arises from oxidation state-dependent hydrogen ion equilibria. It is not possible to assign the sites of these equilibria to specific atoms of the protein based on the available data. In addition to sulfur atoms in the Fe₄S₄S₄C₆ clusters, the titrable sites include amine terminals. The fitted pK values of 7.4 and 8.9 suggest that the sulfur atoms or the NH₂-terminal may be the site of protonation. The characteristic pK values of glutamic and aspartic acid residues and the COOH-terminal are too low. The pK values of tyrosine and lysine residues are high (usually near 10), and thus also are not likely to be the important sites of protonation.

Several arguments favor the sulfur atoms as the probable sites of protonation. Chromatiurn high potential iron-sulfur potential does not exhibit a pH-dependent reduction potential in the range from pH 7 to 11 (4). It is therefore unique among all soluble iron-sulfur proteins for which a study of the pH dependence has been reported. It has been shown by Péisach et al. (17), that the 4Fe-4S center in this protein is solvent inaccessible at pH 7, and therefore could not have sulfur atoms as sites of oxidation state-dependent protonation. Also Job and Bruce (18) have reported a pK of 7.4 for the iron-sulfur core of a water-soluble synthetic analogue of 4Fe-4S protein clusters, with the formula Fe₄S₄(SCH₂CH₂COO)₄⁻. Also, one would expect a hydrogen ion equilibrium at sulfur sites to be intrinsically oxidation state-dependent because of increased charge on reduction of the iron-sulfur center.

Evidence of cluster protonation is not seen in the nmr or epr spectra at low pH (<7), but consideration of the nature of these spectra indicates that the effect of the protonation may not be observed. For example, if the site of protonation is a cysteinyl sulfur atom, the nmr resonance positions of only half the β-cysteinyl proton resonances are resolved, and therefore the protonation of the cysteinyl-sulfur atom would not necessarily be detected. In the epr spectrum, hyperfine interaction between a proton bound to the cysteinyl-sulfur and the unpaired electron spin would be present, but may be too small to be visible in a frozen solution spectrum with significant g value anisotropy.

The evidence presented in this paper, in conjunction with the generally observed negative dependence of Eₚₚ, on pH, indicate that oxidation state-dependent hydrogen ion binding may be a general feature of iron-sulfur protein chemistry. Indeed, it is possible that the enzymatic activity exhibited by hydrogenase and the involvement of iron-sulfur centers in energy conservation at Site I in mitochondria are specializations of such a general feature.

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Origin of the pH dependence of the midpoint reduction potential in Clostridium pasteurianum ferredoxin:oxidation state-dependent hydrogen ion association.
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J. Biol. Chem. 1982, 257:3506-3509.

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