Redox Properties of Several Bacterial Ferredoxins Using Square Wave Voltammetry*

(Received for publication, April 3, 1990)

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The equilibrium reduction potential of the 2[4Fe-4S] ferredoxin (Fd) isolated from four different bacterial strains was determined at a methyl viologen-modified gold electrode using square wave voltammetry. The observed reduction potential at pH 8 for Clostridium thermoacetica Fd was -385 mV; Clostridium pasteurianum, -393 mV; Clostridium thermosaccharolyticum, -408 mV; and Chromatium vinosum, -460 mV versus normal hydrogen electrode at 25 °C. The reduction potential of the C. pasteurianum Fd was found to be pH independent from pH 6.4 to 8.7, indicating that the electron transfer mechanism does not involve proton exchange. In contrast, the reduction potential of the C. thermoacetica Fd was found to be pH dependent from pH 6.4 to 6.7, with \( pK_a \approx 7 \). The +30 mV change in reduction potential from pH 6.7 to 6.4 was attributed to an electrostatic interaction between the iron-sulfur cluster II and the protonated histidine 2 residue located about 6 Å away. The C. vinosum Fd interacted reversibly at the methyl viologen-modified gold electrode, and its reduction potential was verified using visible spectroelectrochemistry. The reduction potential of C. vinosum Fd was found to be 30 mV more positive than previously reported. The similarities of the bacterial Fd reduction potentials are discussed in terms of the homology of their primary structure as reflected by the similarities in the visible and circular dichroic spectra.

The 2[4Fe-4S] ferredoxins (Fds)1 are electron transfer proteins with essential roles in the redox chemistry of photosynthetic and fermentative bacteria. Since a number of natural variants and synthetic analogs are available, this relatively uncomplicated protein provides an excellent model for determining the indirect effects of protein structure on the [4Fe-4S] cluster. From comparisons of native and model analog [4Fe-4S] clusters, it has been suggested that the indirect or extrinsic effects of the polypeptide may significantly modulate the reduction potential of the cluster (1).

The iron-sulfur clusters of Fds have highly conserved structural features and magnetic environments since they are in contact primarily with backbone amides of their single poly- peptides, with the majority of the amino acid side chains facing towards the exterior solvent. When tyrosine 2, an aromatic residue about 6 Å from cluster II in the Clostridium acidiurici Fd was chemically substituted with other amino acids, the overall reduction potential remained the same, even when the aromatic residue was replaced with leucine (2). Only the histidine 2 substitution resulted in a stable derivative that had a measurable 15 mV increase in reduction potential near pH 7. In contrast to Fds, the reduction potentials for high potential iron-sulfur proteins are apparently modulated by various amino acid side chains in close proximity to the cluster (3). For example in Thiocapsa roseopersicina high potential iron-sulfur protein, the reduction potential and the circular dichroic spectra are different at pH 7 and 9; these differences were attributed to histidine 49 (Chromatium vinosum numbering) which is near the lone [4Fe-4S] cluster.

If the reduction potential is found to be pH dependent, then the electron transfer process may be linked to the exchange of a proton. For Clostridium pasteurianum ferredoxin, it is unclear if a proton-linked electron transfer mechanism is involved despite the numerous equilibrium redox studies done as a function of pH (4–10). When using hydrogenase to equilibrate the solution to an ambient potential, the observed reduction potentials for the C. pasteurianum Fd at pH 7 ranged from -371 to -423 mV, and the \( \Delta E^0/\Delta pH \) unit ranged from 0 to -30 mV/pH unit (4–8). Stombaugh et al. (6) have noted the potential change with pH was not sufficiently large to be attributed to an obligatory proton-linked redox mechanism. When using spectroelectrochemical techniques, the reduction potential of C. pasteurianum Fd was found to be pH dependent using circular dichroic spectroscopy (9), and pH independent using EPR spectroscopy (10). Additionally, in kinetic studies for C. pasteurianum Fd, the oxidative electron transfer rates were determined to be pH-independent (11), while the reductive electron transfer rates were pH-dependent (12). The reported pH-dependent reduction potential and electron transfer rates of C. pasteurianum Fd are unusual considering the electron paramagnetic resonance, circular dichroic, and nuclear magnetic resonance spectra of C. pasteurianum Fd were determined to be pH-independent (7).

Since the pH/reduction potential studies of C. pasteurianum Fd are ambiguous, and since Clostridium thermoaceticum Fd has a naturally occurring histidine 2 located near the cluster, we have chosen to examine the reduction potentials of these Fds as a function of pH using an independent analytical method, direct electrochemistry. In previous work using cyclic voltammetry, direct electrochemistry at a methyl viologen-modified gold electrode was used to obtain the reduction potential of spinach ferredoxin at pH 7.1 (13). The reduction potentials for two ferredoxins, including the C. pasteurianum Fd, were obtained using the same methyl viologen-modified gold electrode through differential pulse and cyclic voltammetry at pH 7.6 (14). In the work presented...
here, a methyl viologen-modified gold electrode was used to determine the reduction potential of two *Clostridium* Fds as a function of pH as well as the reduction potential of *Clostridium thermoaceticum* and *Ch. vinosum* Fds by square wave voltammetric experiments. The use of different electrode modifiers is also examined in this paper. This work is one of the first applications of square wave voltammetry to biological molecules.

**Square wave voltammetry (SWV)** has been shown to be a fast, direct, and sensitive method for determining equilibrium reduction potentials of inorganic complexes (15). This experimental approach is not limited by small or complex spectroscopic changes which need to be deconvoluted further in order to determine equilibrium reduction potentials. Nor does it depend upon establishing and maintaining an accurate ambient potential in the bulk solution with either enzymes or dye mediators. Such spectroscopic techniques can be technically cumbersome, especially in negative potential regions where oxygen leakage causes the ambient solution potential to drift.

**MATERIALS AND METHODS**

**Protein Preparation**—*C. pasteurianum* (ATCC 6013) and *C. thermoaceticum* (ATCC 7956) were cultured according to Rabinowitz (16) and Mercer and Vaughn (17), respectively, at the University of Wisconsin-Madison and then grown in a 350-liter fermentor at the University of Wisconsin-Madison, Department of Biochemistry. The Fds were isolated anaerobically at 4 °C using slight modifications of methods previously described (16). *C. thermoaceticum* Fd was a gift from Dr. Stephen W. Ragsdale, Department of Chemistry, University of Wisconsin-Madison. *Ch. vinosum* was grown, and the Fd was purified according to the method of Homer and Tomlinson (18). The proteins were dialyzed anaerobically in the appropriate buffer to and from the electrode surface limit the magnitude of the observed current. When the equilibrium established at the electrode is not limited by electrode kinetics, as in a reversible redox process, the current/time relationship is

\[
i = \frac{nFADC^+}{v} \times \frac{C^+}{C^0}
\]

where \( n \) is number of electrons transferred/reaction site/molecule, \( F \) is Faraday's constant, \( D \) is the diffusion coefficient of the Fd, and \( C^+ \) is the bulk concentration of the Fd. When applying Equation 1 to square wave voltammetry, \( i \) versus \( v \) is linear if diffusion control and rapid equilibrium conditions exist. The current/time relationship, normalized for concentration, is

\[
i = \frac{nFAD}{v} \times \frac{C^+}{C^0}
\]

**Buffers and Redox Mediators**—Appropriate buffers were chosen for the specific pH used in the determination of the equilibrium reduction potentials: cacodylic acid, pH 6.4; phosphate, pH 7.0; Tris, pH 7.3; and pyrophosphate, pH 8.7. The concentration of the buffers was 25 to 50 μl of approximately 0.6 mm protein. A microcell of this size permitted the use of a small amount of protein. Nitrogen bubbled through alkaline pyrogallol, then through water, was continuously passed over the sample solution. No reduction in sample volume from evaporation was observed during the course of the experiments. Experiments at pH 8.0 were carried out in a cell containing a sample volume of 2.0 ml. The solution was made anaerobic by passing a vacuum and purified argon over a heated column of R3-11 catalyst (Chemical Dynamics Corp., South Plainfield, NJ). All cells were water jacketed, and maintained at a constant temperature of 25 °C with a circulating water bath (Lauda/Brinkman model K-4/R/T).

**Square Wave Voltammetry**—In square wave voltammetry, a potential is applied over time in the form of a square wave superimposed on a stair case (see Fig. 1). The applied potential is progressively stepped in fixed increments, \( E_s \) (the step potential), and pulsed anodically and cathodically at each step, \( E_p \) (the pulse potential). The resultant current is measured at the end of each pulse. For each applied potential step, the difference in current is calculated between the anodic and cathodic current generated from the applied potential pulses. For a reversible redox couple, the maximum net current or peak current, \( i_p \), coincides with the equilibrium reduction potential, \( E^0 \). The time between the applied steps, \( \tau \), is the inverse frequency of the applied step potential. The optimal resolution, where the ratio of the peak width at half height (\( W_{1/2} \)) to the peak current is at a maximum, is defined by \( E_p = 20 \text{ mV} \), \( E_s = 50 \text{ mV} \), and \( \tau = 5-2000 \text{ Hz} \). At these frequencies and over the potential range covered, the entire experiment is completed in 5-100 s. In every experiment, voltammograms of buffer, mediator/buffer, and protein/mediator/buffer were obtained in order to determine their individual contributions to the total current.

**Kinetics of Electrode Reactions**—When a potential is applied to an electrode in an unstirred solution, the change in concentration of oxidized and reduced electron accepting species and the diffusion of these species to and from the electrode surface limit the magnitude of the observed current. When the equilibrium established at the electrode is not limited by electrode kinetics, as in a reversible redox process, the concentrations of species at the electrode surface depends only on thermodynamics, i.e., their equilibrium established by the applied potential at the electrode. The current-time response under the appropriate applied potential then follows the Cottrell equation

\[
i = \frac{nFAD}{v} \times \frac{C^+}{C^0}
\]

where \( n \) is number of electrons transferred/reaction site/molecule, \( F \) is Faraday's constant, \( D \) is the diffusion coefficient of the Fd, and \( C^+ \) is the bulk concentration of the Fd. When applying Equation 1 to square wave voltammetry, \( i \) versus \( v \) is linear if diffusion control and rapid equilibrium conditions exist. The current/time relationship, normalized for concentration, is

\[
i = \frac{nFAD}{v} \times \frac{C^+}{C^0}
\]

**FIG. 1.** Time/potential waveform for square wave voltammetry, showing the step potential, \( E_s \), the pulse potential, \( E_p \), and the time between the applied steps, \( \tau \).
$i_p$ versus $\gamma^{-1}$, thus provides an indicator of the extent to which electrode kinetics affects the equilibrium at the electrode.

Spectroelectrochemistry—The spectroelectrochemical determination of the $E'$ of Ch. vinosum Fd was carried out using previously described methods (23) using methyl viologen as the redox dye mediator. A 3-mL solution contained 33 mM Fd, 20 mM methyl viologen, 0.5 M NaCl, and 50 mM Tris, pH 8. Different potentials were applied to a gold foil working electrode of the electrochemical cell. When equilibrium was reached at each potential after about 3 h, the ambient solution potential was verified potentiometrically between the gold foil as the indicator electrode and a saturated Ag/AgCl as the reference, both within the cell. If the measured potential drifted more than $1 \mu V$ min$^{-1}$, it was assumed that there was sufficient oxygen present from leakage to cause the ambient potential to drift and the solution was again purged of any residual oxygen and the experiment continued. 

The $[F_d]/[F_{dd}]$ at each poised potential was determined from the spectroscopic changes measured at 425 nm. All UV visible spectra were obtained with a computer interfaced Cary 219 spectrophotometer.

Electrostatic Calculation—Based on the x-ray crystal structure of P. aerogenes Fd which has a similar sequence, histidine 2 in C. thermosaccharolyticum Fd should be parallel to the face of cluster II and about $6 \pm 1$ A away (24). Using Coulomb's law, the electrostatic potential can be calculated for bringing an electron to cluster II (i.e. a one electron reduction) as influenced by the electrostatic interaction of the electron with the protonated histidine 2. The work required to bring an electron from an infinite distance to cluster II was calculated with the following equation:

\[
U = \sum k q / r
\]

where $\Sigma$ is the electrostatic potential energy (Nm/C or V), $k$ is a proportionality constant ($8.99 \times 10^{-10}$ Nm/C), $q$ is a point charge ($1.6 \times 10^{-19}$ C), $\epsilon_0$ is the dielectric of water (78.5), and $r$ is the distance from histidine 2 to cluster II ($6 \times 10^{-8}$ m). Histidine 2 should not significantly affect the electrostatic potential energy is proportional to $1/r$. The electrostatic work required to reduce cluster II as influenced by the protonated histidine 2 was calculated as $30 \pm 7$ mV.

Simulation of $E'$ versus pH—The change in reduction potential for C. thermosaccharolyticum Fd with pH should follow a sigmoidal curve reflecting the $pK_o$ and $pK_{red}$ of histidine 2, where the $pK$s are the equilibrium constants for the proton association of histidine 2 to the oxidized and reduced forms of Fd. The equlibria are represented as follows.

\[
F_{dd} + c^- \rightleftharpoons F_d + H^+ + e^- \rightleftharpoons F_{dd} - H^+
\]

**SCHEME 1**

The pH dependence of the reduction potential in mV is incorporated into the Nernst equation in the following expression (7).

\[
E = E'_d - 59 \log \frac{[F_{dd}] ([1 + K_{red}[H^+])]}{[F_d]([1 + K_o[H^+]])}
\]

From this equation it can be shown that the magnitude of the change in the observed reduction potential with pH is $59(pK_o - pK_{red})$ mV. Therefore, based on the calculated 30 mV of electrostatic work required to reduce cluster II near the protonated histidine 2 for C. thermosaccharolyticum Fd, a $\Delta pK_{red} = 0.5$ is expected between $pK_o$ and $pK_{red}$.

RESULTS

Redox Potentials—The individual contributions to the total and peak current were determined for buffer, mediator/buffer, and protein/mediator/buffer. An increased current is observed for buffer alone at potentials exceeding the overpotential for hydrogen at the working electrode. A voltammogram is observable for the 0.15 mM methyl viologen solution. However, only a small fraction of the total observed current for protein/mediator/buffer experiments resulted from the methyl viologen in the bulk solution which is not polymerized to the electrode surface. Since the ratio of the concentration of Fd redox centers to methyl viologen was maintained at 10, the peak current in the resulting voltammograms reflected only the reduction potential for Fd. Bianco et al. (14) have also used a large excess concentration of protein to methyl viologen in order to obtain the reduction potential of two Fds.

At 25 °C the formal reduction potential versus NHE of C. pasteurianum Fd was determined as $-390$ mV at pH $6.4$; $-400$ mV at pH $7.0$; $-393$ mV at pH $8.0$; and $-399$ mV at pH $8.7$. The voltammograms of C. thermosaccharolyticum Fd at pH extremes and the plot of reduction potential versus pH is shown in Fig. 2, A and B, respectively. A simulation based on Eq. 2 using an $E'_d = -371$ mV, $pK_o = 7.0$, and $pK_{red} = 7.8$ is shown in Fig. 2B. Additional reduction potentials were not obtained at pH extremes due to the instability of the Fd at these extremes.

The square wave voltammograms of three different ferredoxins at 25 °C in 0.1 M NaCl and 50 mM Tris, pH 8: C. pasteurianum Fd (−396 mV), C. thermosaccharolyticum Fd (−408 mV), and Ch. vinosum Fd (−460 mV) are shown in Fig. 3. In Fig. 4, the measured peak currents, $i_p$ versus $\gamma^{-1}$ for Ch. vinosum Fd are presented. The simulation of the forward and reverse current and the net current for the reversible voltamograms was calculated with the following equation (7).

\[
E = E'_d - 59 \log \frac{[F_{dd}] ([1 + K_{red}[H^+])]}{[F_d]([1 + K_o[H^+]])}
\]

**FIG. 2.** A, square wave voltammograms at the planar gold electrode of 0.9 mM C. thermosaccharolyticum Fd at pH 6.4 and 8.7 in 0.15 mM methyl viologen and 0.1 M NaCl. The applied potential waveform was $E_c = 50$ mV, $E_a = 5$ mV, and $\gamma^{-1} = 25$ Hz. B, the reduction potential versus pH for C. thermosaccharolyticum Fd under the same experimental conditions as A. The solid line is a calculated fit using an $E'_d = -371$ mV, $pK_o = 7.0$, and $pK_{red} = 7.8$ (see "Materials and Methods").

**FIG. 3.** Square wave voltammograms at the planar gold electrode of 0.5 mM C. pasteurianum Fd (A), 0.7 mM C. thermosaccharolyticum Fd (B), and 0.7 mM C. thermosaccharolyticum Fd in 0.15 mM methyl viologen, 50 mM Tris, pH 8, and 0.1 M NaCl (C). The applied potential waveform was $E_c = 50$ mV, $E_a = 5$ mV, and $\gamma^{-1} = 25$ Hz.
Role of Histidine 2 in the Equilibrium Reduction Potential of Ferredoxins

FIG. 4. Dependence of the peak current, \( i_p \), on the square root of the inverse frequency of the applied potential pulse, \( r^{-1/2} \), for 0.7 mM \( Ch. \) vinosum Fd in 0.15 mM methyl viologen, 50 mM Tris, pH 8, and 0.1 M NaCl. The applied potential waveform was \( E_p = 50 \) mV and \( E_r = 5 \) mV.

FIG. 5. Experimental points (*) for the net current (A) and the forward and reverse currents (B) for 0.7 mM \( Ch. \) vinosum Fd in 0.15 mM methyl viologen, 50 mM Tris, pH 8, and 0.1 M NaCl. The applied potential waveform was \( E_p = 50 \) mV, \( E_r = 5 \) mV, and \( \tau^{-1} = 25 \) Hz. The solid line is the current calculated from the theoretical equation for the square wave voltammogram.

To verify the accuracy of the SWV method at the modified gold electrode, the \( E^{\circ} \) of \( Ch. \) vinosum Fd was also determined spectroelectrochemically and found to be identical to the electrochemically obtained value of \(-460 \) mV. Spectroelectrochemical results are shown in Fig. 7 and the inset is the Nernst plot for the experimental data. The slope of the Nernst plot was 65 mV indicating that one electron was transferred per cluster.

We also show in this paper that the electron mediator need not be limited to methyl viologen. When \( Ch. \) vinosum Fd was added in a 10-fold excess to a solution of DMDPP (\( E^{\circ} = -487 \) mV), the observed reduction potential of the Fd was \(-460 \) mV (voltammogram not shown), the same \( E^{\circ} \) obtained through the spectroelectrochemical titration and SWV at the methyl viologen modified gold electrode. Further characterization of the gold electrode/DMDPP mediator is necessary in order to demonstrate if the electrode is modified. However,

<table>
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<tr>
<th>Species</th>
<th>( E^{\circ} ) this work (mV)</th>
<th>( E^{\circ} ) previous work (mV)</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Methyl viologen</td>
<td>-446</td>
<td>-446</td>
<td>31</td>
</tr>
<tr>
<td>DMDPP</td>
<td>-400</td>
<td>-487</td>
<td>19</td>
</tr>
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<td>( C. ) thermoaerophilum</td>
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<td>-366</td>
<td>27</td>
</tr>
<tr>
<td>( C. ) blasiaceticum</td>
<td>-402</td>
<td>-402</td>
<td>27</td>
</tr>
<tr>
<td>( C. ) thermosaccharoliticum</td>
<td>-408</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( Ch. ) vinosum</td>
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<td>-490</td>
<td>9</td>
</tr>
<tr>
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<td>-434</td>
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</tr>
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</tr>
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<tr>
<td>( C. ) thermocellum</td>
<td>-430</td>
<td>-430</td>
<td>14</td>
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* pH 8.

\(^{b}\) pH 7.

Fig. 6. Cyclic voltammograms at the planar gold electrode of 1 mM \( C. \) pasteurianum Fd (solid line) (A) and 0.75 mM \( C. \) thermoaerophilum Fd (dashed line) (B) in 0.2 mM methyl viologen, 50 mM Tris, pH 8, and 0.1 M NaCl. The applied potential was 50 mV/s.

Table I

FIG. 7. Spectroelectrochemical titration of 33 \( \mu M \) \( Ch. \) vinosum Fd in 20 \( \mu M \) methyl viologen, 50 mM Tris, pH 8, and 0.5 M NaCl. The inset is the Nernst plot calculated from the [Fd\textsubscript{red}]/[Fd\textsubscript{ox}] obtained from the absorbance at 420 nm.
In a well-designed electrochemical cell, the redox potential for this Fd is more positive than the previously reported $E^\circ = -490$ mV (9). These results indicate the current is diffusion controlled for Ch. vinosum Fd. They are also in agreement with differential pulse polarographic experiments by Bianco et al. (14), who showed that the current response to increasing concentrations of C. pasteurianum Fd was linear, thus indicating direct electron transfer between the electrode and ferredoxin was taking place.

The methyl viologen-modified gold electrode is experimentally less demanding compared with other direct electrochemical methods for redox proteins. For example, other electrodes (e.g. pyrolytic graphite) require extensive electrode preparation, exacting experimental conditions usually involving promoters, and required continuous cycling over a potential region in order to obtain persistent and reproducible results (25). In addition, our preliminary results indicate that the gold electrode can be used with other organic dye mediators thereby extending its potential range.

**Nernstian $n$**—Theoretically in SWV the peak width at half-height is 126/n mV for a reversible redox couple. If the rate of the applied potential is faster than the rate of electron transfer between the electroactive species and the electrode, then in most cases the observed $n$ is smaller than the theoretical $n$ due to the loss of analytical current. In our experiments, a value of one electron transferred/redox site was observed for all Fds as determined from the peak width at half-height of the experimental voltammograms (See Figs. 3 and 4).

In the case of bulk electrolysis spectroscopic experiments, if the slope of the Nernst plot is not 60/n mV within experimental error then the Nernst equation is either not applicable or inappropriately used. Deviations from the theoretical value could also mean for example that equilibrium of the bulk solution was not reached, or fully oxidized, and reduced species were not obtained. Prince and Adams (10) have proposed that one problem with using hydrogenase to establish ambient potentials is that at the partial pressures and pH values used in the majority of these experiments, it may not be possible to fully reduce the Fd. This in turn affects the apparent ratio of $[\text{Fd}^+]/[\text{Fd}^2-]$ used in the Nernst equation since the concentration of reduced ferredoxin may have been underestimated.

Besides an accurate determination of $[\text{Fd}^+]/[\text{Fd}^2-]$, the importance of determining an accurate value of $n$ was one of the initial reasons for the ambiguity in the reduction potential dependence on pH for Fds. Specifically, Eisenstein and Wang (26) noted a fundamental difference between a two electron transfer to a single redox site and a single electron transfer to two individual redox sites. Their observation required using the appropriate value of $n = 1$ in the Nernst equation. In SWV, the value of $n$ and the reduction potential are determined independently but directly from the peak shape and position of the voltammogram, respectively.

**Reduction Potentials Versus pH**—The extent an ionizable residue participates in an enzymatic or electron transfer reaction is often determined by a reaction rate versus pH profile. However in electron transfer reactions, if the reduction potential also changes with pH, then the change in the driving measurements must be made to verify that the ambient potential is stable. In SWV the experiment is completed in a matter of seconds, and therefore oxygen leakage poses less of a problem. Also, the peroxides generated as well as the stirring of the bulk solution can damage sensitive proteins in bulk electrolysis experiments.

**Evidence of Direct Electrochemistry**—In Fig. 4, it is shown that the peak current is directly proportional to the square root of the inverse frequency of the applied potential. These results indicate the current is diffusion controlled for Ch. vinosum Fd. They are also in agreement with differential pulse polarographic experiments by Bianco et al. (14), who showed that the current response to increasing concentrations of C. pasteurianum Fd was linear, thus indicating direct electron transfer between the electrode and ferredoxin was taking place.

The methyl viologen-modified gold electrode is experimentally less demanding compared with other direct electrochemical methods for redox proteins. For example, other electrodes (e.g. pyrolytic graphite) require extensive electrode preparation, exacting experimental conditions usually involving promoters, and required continuous cycling over a potential region in order to obtain persistent and reproducible results (25). In addition, our preliminary results indicate that the gold electrode can be used with other organic dye mediators thereby extending its potential range.
force of the reaction (the potential difference between the donor/acceptor pair) must be considered when interpreting the kinetic data.

In this study, the reduction potentials for *C. pasteurianum* Fd at pH values of 6.4, 7.0, 8.0, and 8.7 were all -396 ± 5 mV versus NHE at 25 °C, and we conclude that the reduction potential of *C. pasteurianum* Fd is pH independent. Recently, Prince and Adams (10) using EPR spectroscopy also determined the reduction potentials of Fd to be pH independent by buffering the ambient solution potential with methyl viologen. Although this elegant paper showed that the reduction potential of *C. pasteurianum* Fd is pH independent, not all other independent methods corroborated these findings (4, 9).

In this paper, we have demonstrated with a reliable independent method that the reduction potential is indeed pH-independent. In addition, since there are no amino acid residues in the *C. pasteurianum* Fd sequence which have a pKₐ near the pH region investigated and no conformational changes were observed spectroscopically over this same region (7), it is not surprising that the electron transfer mechanism of *C. pasteurianum* Fd is not dependent on the exchange of a proton.

In contrast, the reduction potential of the *C. thermosaccharolyticum* Fd was found to be pH dependent. Based on (a) the magnitude of the observed change in the reduction potential as a function of pH (see Fig. 2A), (b) the simulated reduction potential with pH (see Fig. 2B), and (c) the electrostatic calculation (see “Materials and Methods”), we propose the observed change in reduction potential with pH is due to the electrostatic interaction of histidine 2 with cluster II. On the basis of the work presented in this paper, we expect all Fds with histidine 2 to have pH-dependent reduction potentials. This explains for example the observed E° = -365 mV at pH 7.1 (27) and -385 mV at pH 8 in this work for *C. thermosaccharolyticum* Fd. In addition, it explains the observed +15 mV change near pH 7 in the reduction potential of the histidine 2 modified *C. acidurici* Fd (2).

Histidine at position 2 has been found in heat stable ferredoxins, including Fds from *C. thermosaccharolyticum* (28), *C. tartarivorum* (29), and *C. thermoaceticum*. Although the physiological significance of histidine 2 is not known, it is unlikely that histidine 2 is involved in a pH-regulated control mechanism since ferredoxin itself is not involved in rate limited or committed steps in biological pathways and can be replaced by flavodoxin under low iron concentrations in the media (30). Therefore, we conclude that for *C. thermosaccharolyticum* Fd, the pH-dependent reduction potential is simply due to an electrostatic interaction between cluster II and histidine 2. Moreover, histidine 2 may have additional significance in structural stability of the cluster in thermal stable ferredoxins.

**Reduction Potentials of Various Fds**—From the observed reduction potentials for the various ferredoxins (See Table I), it is evident that changes in amino acid sequence results in small changes in the observed reduction potential. Although SWV and spectroelectrochemical techniques can clearly detect differences between the reduction potential of different Fds, none of these techniques can clearly distinguish small differences in reduction potential of the two clusters within the same Fd. In this paper we show the reduction potentials of three Clostridial Fds are similar to that of the Ch. dioxogen Fd, which has 81 amino acids (9600 D) compared with the 55 amino acids (6300 D) of most Clostridial Fds. Additional evidence is provided, through the similarities in the visible and CD spectra shown in Figs. 8 and 9, that the cluster environment is essentially the same for the different ferredoxins examined. The visible and CD spectra for *C. thermosaccharolyticum* Fd were previously unreported. Since the majority of amino acid side chains are directed toward solvent and the clusters are surrounded by backbone amides, it is reasonable that the reduction potentials for these ferredoxins are very similar.

We have shown that despite the apparently strong conservation of the internal environment of low potential iron-sulfur clusters, the reduction potential of a ferredoxin can be altered significantly by the extrinsic effect of a single amino acid side chain in close proximity of the cluster. More specifically, an observed 30 mV change in reduction potential of the *C. thermosaccharolyticum* Fd with pH is probably due to the protonation of histidine 2 located near the iron-sulfur cluster II.

**Acknowledgments**—We would like to thank Dr. M. J. Benecky, Mount Sinai Hospital, Milwaukee, WI for his helpful discussions and expert assistance in obtaining the CD spectra.

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