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Oxidation–Reduction Properties of Several Low Potential Iron–Sulfur Proteins and of Methylviologen†

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ABSTRACT: Apparent oxidation–reduction potentials at pH 7.0 and 25 °C were determined using the H_2–hydrogenase system with ferredoxins from the following sources: *Clostridium pasteurianum,* −403 mV; *C. tartarovorum,* −424 mV; *C. acidi-urici,* −434 mV; *Peptococcus aerogenes,* −427 mV; *Chromatium* D, −482 mV (pH 8.0); *B. polymyxa,* Fd I, −377 mV, and Fd II, −422 mV; and spinach, −428 mV. The pH dependence of these values was variable, ranging from −2 to −24 mV/pH unit increase for different ferredoxins. Over the range of buffer concentrations between 0.05 and 0.2 M, the potentials did not vary significantly. The number of electrons transferred during reduction (as determined by integrations of EPR spectra and by dithionite titration) is 2 for the first five proteins, while potentiometric data for all the cases fit a Nernst equation for which $n = 1$. The $E°'$ value for the redox indicator methylviologen at pH 7.4 was found to be −460 mV, according to both the H_2–hydrogenase system and cyclic voltammetry, significantly different from the value previously reported at higher pH's. Additionally, the presence of *C. pasteurianum* ferredoxin appears to shift the $E°$ value of methylviologen to even more negative values. An analysis of sources of error inherent with potential determinations with H_2 and hydrogenase is presented. The electronic and EPR spectra of *P. aerogenes* ferredoxin, for which the x-ray structure has been published, are given here. It appears that the determination of potentials of ferredoxin and other low-potential proteins with the H_2–hydrogenase system affords certain experimental advantages over alternative methods currently employed with these and similar substances.

Among the iron–sulfur proteins, two distinct classes of these proteins transfer electrons at oxidation–reduction potentials near the potential of the H_2/H^+ couple. These classes are (a) the plant-type ferredoxins, which contain a cluster of two nonheme iron and two labile sulfur atoms per molecule, and (b) the bacterial-type ferredoxins, which appear to contain one or two clusters of four iron and four labile sulfur atoms per molecule. The distinguishing characteristics of these proteins have been extensively reviewed (see Orme-Johnson, 1973, and references therein). In particular, the amino acid sequences of many of these proteins are known, several crystallographic structure studies on the iron–sulfur proteins are near the atomic resolution stage, and a number of synthetic analogues of the metal clusters

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have been prepared. To those interested in the detailed relationships between the structure and the physical properties of these proteins, accurate values of the physical parameters, or rather of the relative values of these parameters within and between classes of these proteins, are important.

There have been a number of reports describing measurements of the oxidation–reduction potentials of ferredoxins. Tagawa and Arnon (1968) observed that, as measured by equilibration of the proteins with the H_2/H^+ couple, the potentials of spinach and *Clostridium pasteurianum* ferredoxins were −0.42 and −0.39 V, respectively, that these potentials were independent of pH near neutrality, and that the appropriate value of n in Nernst's equation (eq 1) for this process was 1. They concluded that both the plant- and bacterial-type ferredoxins accept a single electron on reduction. That the clostridial ferredoxins are actually two-electron oxidants was shown by Sobel and Lovenberg (1966) using several methods in which substances with known reducing characteristics were brought into equilibrium with *C. pasteurianum* ferredoxins. This conclusion was supported in subsequent studies by Evans et al. (1968) who followed spectrophotometric changes during oxidation of reduced ferredoxin with NADP and methylviologen, by Eisenstein and Wang (1969), who studied the spectrophotometric properties of equilibrium mixtures of ferredoxin and methylviologen, by Mayhew et al. (1969) who carried out spectrophotometric titrations with dithionite, and by Orme-Johnson and Beinert (1969), who noted two kinds of EPR signals arising during reductive titrations of *C. pasteurianum* ferredoxin. It does appear (and the data reported below support this) that the value of n required for Nernst's equation to correctly describe the data is 1, as Tagawa and Arnon suggested. The meaning of this, that two different single-electron centers are present in clostridial ferredoxins, was pointed out by Eisenstein and Wang (1969) and is implied by the EPR result (Orme-Johnson and Beinert, 1969).

Among the methods which might be employed to measure these potentials, electrometric techniques (Wilson, 1969; Hawkridge and Kuwana, 1973; Eisenstein, 1968) may suffer from electrode polarization and poisoning and require the use of mediators, and equilibration with redox active dyes (Clark, 1960; Huang and Kimura, 1973) may involve effects of protein–dye interactions. Because purified preparations of hydrogenase are now available (Nakos and Mortenson, 1971; Haschke and Campbell, 1971), we have chosen to use equilibration of the ferredoxin with the H_2/H^+ couple under conditions where solvent and buffer ion interactions are the only appreciable perturbing influences on the measurement. Reduction was followed spectrophotometrically since the molar absorbancies of the reduced and oxidized ferredoxins differ. We have obtained apparent potentials directly referred to the H_2/H^+ couple and have tried to ascertain the influence of limited variations of pH and ionic strength on the potentials of spinach ferredoxin and several bacterial-type ferredoxins. We also have evaluated sources of experimental error in these determinations and have studied the oxidation–reduction properties of the dye methylviologen, which often is employed in potentiometric measurements with these proteins.

Materials and Methods

Preparation of Ferredoxins. Spinach ferredoxin (A_{420}/A_{280} = 0.47) was prepared according to Borchert and Wessels (1970). *C. acidi-urici* ferredoxin (A_{390}/A_{280} = 0.78) was prepared according to Hong and Rabinowitz (1970). *C. pasteurianum* ferredoxin (A_{390}/A_{275} = 0.80) and *C. tartarovorum* (A_{390}/A_{280} = 0.79) were prepared by the same procedure used for the *C. acidi-urici* protein. *Chromatium vinosum* ferredoxin (A_{385}/A_{275} = 0.66) was a gift from H. Winter. Prior to use, the sample was chromatographed on DEAE-cellulose (Whatman DE-52) in 0.15 M potassium chloride–0.15 M Tris-Cl, pH 8.0. *B. polymyxa* ferredoxins I and II (OD_{395}/OD_{280} = 0.56 and 0.72, respectively) were prepared according to Stombaugh et al. (1973), omitting the final preparative gel electrophoresis step for Fd II. *Peptococcus aerogenes* ferredoxin (A_{390}/A_{280} = 0.78) was a gift from L. Jensen and L. Sieker, and was rechromatographed before use under the same conditions employed with the *Chromatium vinosum* protein.

Preparation of Hydrogenase. A partially purified hydrogenase extract was prepared as a by-product of a procedure for isolation of the nitrogen-fixing enzymes of *C. pasteurianum* (strain W5A). The hydrogenase fraction was eluted from the first DEAE-cellulose column with 0.15 M NaCl and 0.02 M Tris-Cl, pH 7.4) following the procedure of Tso et al. (1972). The crude hydrogenase was further purified by chromatography on Sephadex G-100 in the presence of 50 mM Tris-Cl, pH 7.4, and 10 mM sodium dithionite. The specific activity of the hydrogenase was determined by following the increase in absorbance at 580 nm due to the reduction of 10 mM methylviologen in 20 mM Tris-Cl, pH 7.4, equilibrated with 1 atm of H_2. The specific activity of the final preparation was 60 μmol min^{-1} mg^{-1} in a 1-ml reaction volume.

Description of Apparatus. Prepurified N_2 (Air Reduction Co., Inc., New York, N.Y.) containing about 10 ppm of O_2 was treated with BASF catalyst R3-11 (Chemical Dynamics Corp., P.O. Box 395, S. Plainfield, N.J. 07080) at about 120 °C. The O_2 content of this gas, as well as electrolytic H_2 gas (National Cylinder Gas, Chicago, Ill., treated with a Deoxo catalytic purifier, Engelhard Industries, Newark, N.J.), was maintained at less than 0.5 ppm of O_2 as measured by the technique of Sweetser (1967). Except for the furnace tube for the BASF catalyst, which was constructed of silver-soldered copper tubing (2.5-cm diameter), the gas train was constructed entirely of glass or 1.5-mm (i.d.) seamless copper tubing. All glass-to-glass connections were 7/25 standard tapered joints sealed with Apiezon N or W (Thomas Scientific Apparatus, Philadelphia, Pa.); all glass-to-copper joints were overlapped and joined with Epibond epoxy (Furane Plastics, Inc., Los Angeles, Calif.). Copper-to-copper joints were soft-soldered. All stopcocks were the Brookhaven spring-loaded variety (Eck and Krebs Scientific Laboratory Glass Apparatus, Inc., Long Island City, N.Y.).

A 6-mm bore mercury manometer, the gas trains, and an oil vacuum pump with a liquid-nitrogen-cooled trap were attached to a glass manifold. Also connected to the manifold was a coil of 1.5-mm (i.d.) copper tubing, approximately 10 ft in length. This coil was introduced to minimize the strain exerted upon the manifold by the shaking of a glass cuvette which was attached to the end of the copper coil via a tapered glass joint. A stopcock was inserted between the glass joint and the cuvette so that, after equilibration under the mixture of H_2 and water vapor, the cuvette could be removed readily from the gas train and placed in a spectrophotometer. Another stopcock provided an additional inlet to the cuvette to facilitate the anaerobic addition of hydrogenase with a microliter syringe.

Reduction of Proteins. The cuvette containing 0.02 to

0.06 mM ferredoxin in 0.1 M potassium phosphate (0.1 M Tris-Cl in experiments with *Chromatium vinosum* ferredoxin) was rendered anaerobic by repeated evacuation and flushing with N_2. The ferredoxin concentration was then measured spectrophotometrically. Approximately 30 μg of the partially purified hydrogenase in 0.1 M potassium phosphate buffer (pH 7.4) was added anaerobically in 5 μl to the 3.0 ml of ferredoxin solution.

Increasing partial pressures of H_2 were equilibrated with the hydrogenase–ferredoxin solution by rapid shaking of the cuvette in a Gilson respirometer water bath (Gilson Medical Electronics, Inc., Middleton, Wis.) thermostated at 25.0 °C. A total of five or six different H_2 pressures normally were employed in each experiment. During shaking, the cuvette was mounted in an inclined position to facilitate rapid equilibration of the gas and liquid phases. When the pressure inside the manifold (the sum of the H_2 and water vapor pressures) was observed to be constant (about 15 min of shaking was required), the cuvette was sealed off from the gas train and the absorbance of the solution was determined in a Beckman D U spectrophotometer (Beckman Instruments, Inc., South Pasadena, Calif.) equipped with a Gilford linear absorption converter (Gilford Instruments Laboratory, Inc., Oberlin, Ohio), which had been calibrated with Gilford Model 202 absorbance standards. The cuvette chamber also was thermostated at 25 °C. Temperature variation inside the cuvette while the sample was in the light beam was less then 0.2 °C as measured by placing an Anschutz style thermometer calibrated at 0.0 and 32.0 °C, directly inside the cuvette. The cuvette then was returned to the water bath for an additional 5–10 min of shaking before the absorbance was again determined. This assured that equilibrium had been achieved. Reversibility was checked by performing the last equilibration at a reduced p_{H_2}.

The p_{H_2} was determined manometrically using a cathetometer readable to a tenth of a millimeter. (See discussion of $dE^{\circ\prime}/dp_{H_2}$.) The observed pressure was corrected for the barometric pressure, and for the observed partial pressure of the water vapor, to arrive at the p_{H_2} in the system. The pH of the reaction mixture was determined following the experiment with a Leeds and Northrup miniature pH electrode assembly (Leeds and Northrup Co., Philadelphia, Pa., electrodes No. 117147 and 117145). Primary standards (Bates, 1962) were used to calibrate the meter at pH 4.00, 7.41, and 9.21.

Determination of $E^{\circ\prime}$ for Methylviologen in Presence of C. pasteurianum Ferredoxin. $E^{\circ\prime}$ values for 0.64 mM methylviologen[1] were determined in the presence of 0, 0.023, 0.048, and 0.064 mM *C. pasteurianum* ferredoxin in 0.1 M potassium phosphate (pH 7.45). The extinction coefficients reported by Eisenstein and Wang (1969) were employed in the calculation. These experiments were performed as described above, except that absorbance at two wavelengths (425 and 600 nm) was measured to determine with the following equations the extent of reduction of each species:

$$A_{600} = A_{600}^{ox}\,(1 - 0.08\,([Fd_{red}]/[Fd_{total}])) + 11.4\,[\text{mM MeV}]\,([MeV_{red}]/[MeV_{total}])$$

$$A_{435} = A_{425}^{ox}\,(1 - 0.546\,([Fd_{red}]/[Fd_{total}])) + 1.61\,[\text{mM MeV}]\,([MeV_{red}]/[MeV_{total}])$$

where A_{600} is equal to the absorbance at 600 nm; A_{425}, the absorbance at 425 nm; A_{600}^{ox}, the absorbance at 600 nm of the fully oxidized system; A_{425}^{ox}, the absorbance at 425 nm of the fully oxidized system; [mM MeV], the millimolar concentration of methylviologen; ($[Fd_{red}]/[Fd_{total}]$), the fraction of ferredoxin reduced; and ($[MeV_{red}]/[MeV_{total}]$), the fraction of methylviologen reduced.

Calculation of $E^{\circ\prime}$. The redox potential of each protein was determined from Nernst's equation (eq 1) for the hydrogen and ferredoxin couples in equilibrium.

$$E_{Fd}^{\circ\prime} = \frac{RT}{2F}\ln [H^+]^2 + \frac{RT}{2F}\ln\frac{1}{p_{H_2}^{\circ}} + \frac{RT}{nF}\ln\frac{[Fd_{red}]}{[Fd_{ox}]} \quad (1)$$

which is equal to

$$E_{Fd}^{\circ\prime} = E_{H_2}^{\circ} + \frac{0.0592}{n}\log\frac{[Fd_{red}]}{[Fd_{ox}]} \quad (2)$$

at 25 °C, where $E_{Fd}^{\circ\prime}$ is the redox potential of the protein; ($[Fd_{red}]/[Fd_{ox}]$), the ratio of reduced to oxidized protein; $E_{H_2}^{\circ}$, the potential of the hydrogen electrode; all other symbols have their usual Nernst equation values. By plotting $E_{H_2}^{\circ}$ vs. log ($[Fd_{red}]/[Fd_{ox}]$), $E_{Fd}^{\circ\prime}$ and n values can be determined graphically by least-squares analysis.

The ratio of reduced to oxidized ferredoxin is calculated from the absorbance data and eq 3

$$\frac{[Fd_{red}]}{[Fd_{ox}]} = \frac{OD_i - OD}{OD - OD_i\,(A_{red}/A_{ox})} \quad (3)$$

where OD_i is the absorbance of the fully oxidized material; OD is the absorbance at equilibrium; and (A_{red}/A_{ox}) is the ratio of the molar absorbancy of the molar reduced and oxidized species.

Determination of (A_{red}/A_{ox}). (A_{red}/A_{ox}) was determined for all proteins except *Chromatium vinosum* ferredoxin by measuring the decrease in absorbance at the λ maximum of the ferredoxin (ca. 400 nm for bacterial ferredoxins and 420 nm for the spinach ferredoxin) when a sample of the protein was reacted with a slight excess of solid sodium dithionite at pH 8.0, 7.4, and 6.5. Data also were collected at 425 nm for *C. pasteurianum* ferredoxin at pH 7.45. After measuring the decrease in absorbance, another aliquot of solid dithionite was added to ensure that complete reduction had been obtained and that excess dithionite was not contributing significantly to the absorbance measured for the reduced species. Reoxidation of the protein by exposure to air revealed that the absorbance returned to 95% or greater of the original value, indicating little loss of the chromophore.

An (A_{red}/A_{ox}) value for *Chromatium* ferredoxin could not be obtained by the dithionite technique, because the protein was not reduced by sodium dithionite with or without catalytic amounts of methylviologen. To determine (A_{red}/A_{ox}) for this protein it was necessary to use a computer program which fitted the optical density and p_{H_2} data to the hyperbolic equation:

$$OD = OD_i\frac{1 + (A_{red}/A_{ox})\,K_{eq}\sqrt{p_{H_2}}}{1 + K_{eq}\sqrt{p_{H_2}}}$$

which was derived from the equation for K_{eq} of the hydrogen and ferredoxin couples at a constant pH and from the expression of the absorbance of the solution in terms of the concentrations and extinction coefficients of each of the ferredoxin forms. In the above equation, OD is the optical density at any given hydrogen pressure, OD_i and (A_{red}/A_{ox})

[1] Abbreviations used: Fd, ferredoxin; MeV, methylviologen; NBS, *N*-bromosuccinimide.

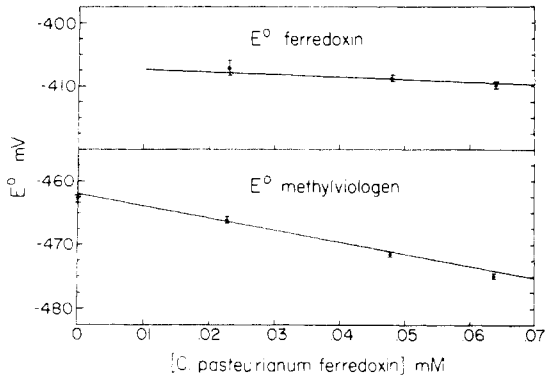

FIGURE 1: The apparent $E^{\circ\prime}$ of 0.64 mM methylviologen in the presence of varying concentrations of *Clostridium pasteurianum* ferredoxin is shown in the bottom half of the figure. The $E^{\circ\prime}$ values for both methylviologen and the ferredoxin were calculated using the extinction coefficients determined in the text by Whitson et al. and the equations given (1973). Standard deviations, calculated as described in Table I, are also shown. The slight positive shift of the apparent $E^{\circ\prime}$ values for *C. pasteurianum* ferredoxin (upper portion of Figure 1) as the concentration of ferredoxin decreases is probably caused by the decreased stability of the ferredoxin at low protein concentrations (see $dE^{\circ\prime}/d([Fd_{red}]/[Fd_{ox}])$. All determinations were done in the presence of 0.1 M potassium phosphate (pH 7.45) and 32 μg of hydrogenase. The sample volume was 3.0 ml.

have their previously defined meanings, K_{eq} is the equilibrium constant at a constant pH, and p_{H_2} is the partial pressure of hydrogen. It was possible to estimate the (A_{red}/A_{ox}) value for *Chromatium vinosum* ferredoxin as 0.82 ± 0.01. This analysis, when applied to some of the other proteins which were not denatured appreciably (<1%) during the experiment, yielded values of (A_{red}/A_{ox}) that agreed well with data collected by the simple dithionite reduction technique.

Electrochemical Determination of $E^{\circ\prime}$ of Methylviologen. Polarography, cyclic voltammetry, and potentiometric titrations were performed in attempts to measure the midpoint potential of methylviologen. The cyclic voltammetric instrument was constructed and operated as described by Whitson et al. (1973). The cell was a water-jacketed (25 °C) voltammetry cell with deaeration by purified N_2. The counter electrode was a platinum wire spiral. The saturated calomel reference electrode (Beckman Instruments, No. 476000) was terminated with a cracked-glass junction. The cyclic voltammetric measurements were performed in 0.1 M potassium phosphate buffer (pH 7.45) with varying concentrations of methylviologen. The scan rate was varied from 25 to 400 mV/s.

The data were analyzed according to the theory for a reversible, one-electron reduction of the viologen as developed by Nicholls and Shain (1964). The half-wave potential, $E_{1/2}$, was evaluated by adding 28.5 mV to the observed cathodic peak potential. This value was assumed to be equal to the standard potential, $E^{\circ\prime}$, although the two are related by $E_{1/2} = E^{\circ\prime} - (RT/nF) \ln (D_o/D_r)^{1/2}$ where D_o and D_r are the diffusion coefficients of the oxidized and reduced methylviologen, respectively. It is assumed here that D_o is equal to D_r.

The polarographic determinations were performed with a dropping mercury electrode with a 2-s drop time. The voltage was scanned from −0.208 to −0.658 V at a rate of 1.5 mV/s. The polarograms were obtained in 0.1 M potassium phosphate, pH 7.45, with 5×10^{-4} M methylviologen.

A potentiometric titration was performed in a sodium phosphate buffer adjusted to pH 11.0 with sodium hydroxide (Bates and Bowen, 1956) with a saturated calomel electrode as reference and three platinum working electrodes (Bates, 1954). A 500-ml vessel contained the platinum electrodes, a saturated calomel electrode, a thermometer, a gas inlet, and a gas outlet, all inserted through a rubber stopper. The solution was rapidly sparged with N_2 prepared as described earlier, while the vessel was immersed in a 25 °C water bath. Through a serum-stopper sealed inlet, 5-μl aliquots of 1.2 M sodium dithionite were added with a Gilmont syringe (Gilmont Instruments, Inc., Great Neck, N.Y., Model No. S-3100) through a 6-in. platinum needle placed below the buffer which contained 0.64 mM methylviologen. Throughout the course of the titration, the potentials of the three platinum electrodes with respect to the reference were read sequentially with a Hewlett-Packard Model 740B DC standard differential voltmeter operated in the "infinite impedence" mode (Service Manual, Hewlett-Packard Co., Loveland, Calif.). The three working electrodes agreed to within 0.1 mV at all times. The data were analyzed as a logistic function by the method of Reed and Berkson (1929).

Results

$E^{\circ\prime}$ Determinations of Various Iron–Sulfur Proteins. $E^{\circ\prime}$ vs. pH data for the ferredoxins are summarized, along with a tabulation of (A_{red}/A_{ox}) values employed in the calculations, and the average η values, in Table I.

Effect of Ionic Strength on $E^{\circ\prime}$ of C. pasteurianum, B. polymyxa Fd I, Chromatium vinosum, and Spinach Ferredoxins. Although minor variations (1–4 mV) in $E^{\circ\prime}$ values were obtained by varying the buffer molarity between 0.2 and 0.05 M, at a constant pH, these variations were on the same scale as the limits to the accuracy of the measurements (see Discussion).

Effect of C. pasteurianum Ferredoxin on $E^{\circ\prime}$ of Methylviologen. The results of experiments designed to determine the midpoint potential of methylviologen in the presence of varying concentrations of *C. pasteurianum* ferredoxin are presented in Figure 1. The midpoint potential of the methylviologen, calculated using the extinction data of Eisenstein and Wang, appears to shift to more negative values as the ferredoxin concentration increases. The slopes of the plots of $E_{H_2}^{\circ}$ vs. log $([MeV_{red}]/[MeV_{ox}]$ were 59.1 ± 0.9 mV/log unit. $E^{\circ\prime}$ for 0.64 mM methylviologen in the absence of any added protein was −0.46 V.

Polarographic Determination of $E^{\circ\prime}$ for Methylviologen. The polarographic curve exhibits a weak absorption component, which results from the nearly equal potential at which both the absorption and reduction wave appears. This component makes it impossible to obtain accurate potential determinations by the polarographic technique.

Cyclic Voltammetric Determination of $E^{\circ\prime}$ for Methylviologen. The $E^{\circ\prime}$ of methylviologen was concentration dependent over the concentration range employed. At 1×10^{-4} M methylviologen, the midpoint potential measured was −464 ± 2 mV; at 5×10^{-4} M, −457 ± 2 mV; and at 2×10^{-3} M, −447 ± 1 mV. These measurements were made at both 25 and 50 mV/s scan rates. The cyclic voltammograms also showed evidence of weak absorption whose net effect is to shift the apparent midpoint potential to slightly more positive values. By plotting the $E^{\circ\prime}$ determined by the method described earlier, as a function of the square root of a varying scan rate and extrapolating to zero scan rate, at which point the error from weak absorption should become

Table I.

Ferredoxin	pH	$E^{\circ\prime}$[a,l]	Estimated $E^{\circ\prime}$[l] (pH 7.0)	Estimated dE°/dpH (mV/pH unit)	n[b]	A_{red}/A_{ox}[k]	Previously Reported $E^{\circ\prime}$[l]
Spinach	7.05	−427.9 ± 0.5	−428	−4	1.08 ± 0.15	0.49 (420)	−0.42 V, pH 6.6–8.2[e]
	7.75	−432.9 ± 0.5					−423, pH 8.0–9.5[j]
	8.20	−431.9 ± 0.2					
B. polymyxa I	6.60	−372.0 ± 0.9	−377	−11		0.66 (395)	−0.39 V[f]
	7.20	−379.5 ± 0.5			1.10 ± 0.12		
	7.75	−382.1 ± 0.5					
B. polymyxa II	6.87	−418.9 ± 0.9	−422	−24	0.95 ± 0.07	0.67 (395)	
	7.20	−429.2 ± 1.7					
	7.75	−438.7 ± 1.2					
C. pasteurianum	6.70	−397.6 ± 1.2	−403	−12	1.06 ± 0.04	0.62 (390)	−335, pH 7.0[j]
	7.18	−407.8 ± 1.2					−0.39 V, pH 6.1–7.4[e]
	7.75	−411.0 ± 1.5					−407, pH 7.0[g]
							−410, pH 7.4[c,h]
C. tartarivorum	7.01	−423.8 ± 0.9	−424				
	7.48	−424 ± 0.8		−3	1.04 ± 0.02	0.68 (390)	
	7.90	−426.4 ± 1.0					
C. acidi-urici	6.98	−434.5 ± 0.3	−434	−2	0.99 ± 0.14	0.64 (390)	
	7.50	−434.6 ± 0.8					
	7.91	−436.8 ± 1.2					
P. aerogenes	6.89	−426.0 ± 0.5	−427	−8	0.96 ± 0.02	0.66 (390)	
	7.30	−431.5 ± 1.3					
	7.65	−430.5 ± 0.8					
Chromatium vinosum	8.10	−481.3 ± 1.7	−482 (pH 8.0)	−11	1.13 ± 0.14	0.82 (385)	−0.49 V, pH 7.0[d,i]
	8.50	−488.0 ± 1.5					−489, pH 9.0[j]
	8.90	−491.4 ± 0.3					

[a] Determined by least-squares analysis of log (reduced/oxidized) vs. $E^{\circ}_{H_2}$ data. Standard errors were determined as the square root of the average squared displacement (in millivolts) from the least-squares line. [b] Determined as the average of at least three determinations from the E°_{H} vs. log (reduced/oxidized) data. [c] Data corrected using $E^{\circ\prime}$ of methylviologen equal to −471 mV (see Figure 1). [d] Data consist of 1 atom of hydrogen pressure equilibration. [e] Tagawa and Arnon, 1968. [f] Yoch and Valentine, 1972. [g] Sobel and Lovenberg, 1966. [h] Eisenstein and Wang, 1969. [i] Bachofen and Arnon, 1966. [j] Ke et al., 1974. In this reference, the number of electrons accepted per mole of protein and the value of n in Nernst's equation are taken as the same quantity. See Eisenstein and Wang (1969) and the discussion in the present paper, which emphasize the difference between these quantities. [k] Numbers in parentheses are wavelengths in nanometers. [l] $E^{\circ\prime}$ in millivolts unless otherwise specified.

negligible, it is possible to estimate that the potentials as reported above, determined at a relatively slow scan rate, are probably 3–4 mV more positive than the true $E^{\circ\prime}$ values. All calculations are based on a potential of −242 mV for the saturated calomel electrode (Skoog and West, 1969). Our electrode was checked against a hydrogen electrode in the pH 11.0 Bates and Bowen buffer and was measured to have a potential of −241 mV at 25 °C. The electrode was found to have a potential of −244 mV in the NBS pH 7.41 buffer (Bates, 1962) at 25 °C.

Potentiometric Titration of Methylviologen. The results of the potentiometric titration of 0.64 mM methylviologen at pH 11.0 indicated that the E° under these conditions was −445 ± 2 mV, in good agreement with values found by Michaelis and Hill (1933) and confirmed by Eisenstein and Wang (1969).

Discussion

An analysis of the error in the $E^{\circ\prime}$ calculations from the experimental determination of the variables T, p_{H_2}, pH, and $[Fd_{red}]/[Fd_{ox}]$ seems appropriate.

$$dE^{\circ\prime}/dT \text{ (from eq 1)}$$

$$\frac{dE^{\circ\prime}}{dT} = 9.9 \times 10^{-5} (\log [H^+]^2 - \log p_{H_2}) + 1.99 \times 10^{-4} \log \left(\frac{[Fd_{red}]}{[Fd_{ox}]}\right) \quad (4)$$

Since p_{H_2} and $[Fd_{red}]/[Fd_{ox}]$ were limited to values near to unity and the pH was usually about 7:

$$\frac{dE^{\circ\prime}}{dT} \simeq 9.9 \times 10^{-5} (-14) \simeq 1.5 \times 10^{-3} \text{ V/°K} \quad (5)$$

Consequently, adequate temperature control must be maintained during the experiment for accurate measurements. The authors' estimates of the amount of error involved in these experiments arising from this error as well as other sources of errors are summarized in Table II.

$$dE^{\circ\prime}/dp_{H_2} \text{ (from eq 1)}$$

$$\frac{dE^{\circ\prime}}{dp_{H_2}} = \frac{0.0129}{p_{H_2}} \frac{\text{V}}{\text{atm of } H_2} \quad (6)$$

The equation indicates that the error from the uncertainty in the p_{H_2} measurement is a function of the total H_2 pressure of the system. Thus errors in E° can be greatly reduced by avoiding extremely low partial pressures of H_2; e.g., an error of 1.0 mmHg in the measurement of the H_2 pressure would result in an error of about 2 mV in the E° calculations at a p_{H_2} of 0.01 atm, but would result in less than 0.5-mV error at 0.04 atm of H_2.

$$dE^{\circ\prime}/dpH \text{ (from eq 1)}$$

$$dE^{\circ\prime}/dpH = -0.0592 \text{ V/pH unit} \quad (7)$$

An error of 0.1 pH unit in the measurement of the pH of the reaction mixture would result in about 6-mV error in

Table II: Experimental Source of Error, Maximum Error in Variables and Resultant Errors in $E^{\circ\prime}$ Values.

Variable	Estimated Maximum Errors in Variable[a]	Resulting Error in $E^{\circ\prime}$[c] (mV)
Temperature	±0.2 K	0.3[d]
pH	±0.02 pH unit	1.2[d]
p_{H_2}	$\pm 1.3 \times 10^{-3}$ atm[b]	0.4[d]
A_{red}/A_{ox}	±0.02	2.5
Decrease in OD_i	3%	2.5

[a] Estimates of the maximum amount of error in experimentally determined variables during a typical experiment. [b] This is the sum of three errors, in the cathetometer and barometer readings, and in the solvent vapor pressure correction. [c] Calculated for a hypothetical experiment in which the p_{H_2} was always greater than 0.05 atm, and the true A_{red}/A_{ox} value was 0.70 and $0.5 \leq ([Fd_{red}]/[Fd_{ox}]) \leq 2$. [d] Errors due to these variables are random in nature. Consequently, these errors will average out over a series of experiments and do not contribute significantly to the experimental uncertainty of $E^{\circ\prime}$.

E°. Therefore, extreme care must be taken during calibration of pH meters, and the use of freshly prepared primary standards is required.

$$\frac{dE^{\circ\prime}}{d([Fd_{red}]/[Fd_{ox}])} \text{ (from eq 1)} = +0.0257 \frac{[Fd_{ox}]}{[Fd_{red}]} = -0.0257 \frac{[Fd_{red}]}{[Fd_{ox}]} \text{ V/unit} \quad (8)$$

The error in determining the ratio of reduced to oxidized ferredoxin comes from two sources. First, any error involved in determining A_{red}/A_{ox} will show up in the final calculation of $E^{\circ\prime}$; if the values measured or assumed by authors were routinely reported, then it would be easy to correct calculated $E^{\circ\prime}$ values based on subsequent spectrophotometric studies. Two literature values for A_{red}/A_{ox} are available which appear to us to be accurate: one for *C. acidi-urici* ferredoxin at 390 nm, $A_{red}/A_{ox} = 0.641$, obtained by Uyeda and Rabinowitz (1971) and another for *C. pasteurianum* ferredoxin determined at 425 nm by Eisenstein and Wang (1969), $A_{red}/A_{ox} = 0.454$. The data collected here with the solid dithionite technique, 0.64 ± 0.01 and 0.45 ± 0.01 for *C. acidi-urici* and *C. pasteurianum* ferredoxins, respectively, are in reasonable agreement with the above values.

A second source of error involved in the determination of the ratio of reduced and oxidized ferredoxin is the denaturation of the protein during the experiment. Since the calculation of the ratio of reduced to oxidized species depends on the absorbance of the initial fully oxidized protein (eq 3), any loss of chromophore after this initial (absorbance) measurement will make the protein appear more easily reducible than it actually is; this results in the calculated E° value being more positive than the true value. Unfortunately, this type of error is difficult to eliminate or to compensate for accurately in calculations. Even under the anaerobic conditions attainable with the type of apparatus used, a small (1–3%) loss of chromophore from protein denaturation seems to be unavoidable with the more labile ferredoxins. Denaturation may result in part from inactivation at the gas–liquid interface of bubbles formed during phase equilibration. Correction for this error is difficult because the final ferredoxin concentration, in a partially denatured sample, is hard to determine accurately. Attempts to re-oxidize the protein by evacuating H_2 from the system, so that the final ferredoxin concentration can be measured optically, results in concentrating the protein by loss of water vapor during evacuation. Re-oxidizing the protein with O_2 to determine the ferredoxin concentration is inadvisable, because O_2 may denature the partially reduced ferredoxin (Petering et al., 1971).

Errors in $E^{\circ\prime}$ calculations contributed by these difficulties can be greatly reduced by the judicious selection of experimental conditions. First (eq 8), keeping the ratio of reduced to oxidized ferredoxin close to unity ($\frac{1}{2} \leq [Fd_{red}]/[Fd_{ox}] \leq 2$), decreases the error from the uncertainty in the ratio of reduced to oxidized protein. Working within this relatively restricted range reduces the error in $E^{\circ\prime}$ calculations arising both from the error in determination of A_{red}/A_{ox} and from the loss of chromophore. Second, working as rapidly as possible, consistent with attainment of equilibrium, and rigorously eliminating leaks in the manometric system minimize the problem of protein denaturation. The application of spectropolarometric detection of redox state (Ke et al., 1974) to the H_2/hydrogenase method might alleviate some (but not all) of the problems encountered here.

Summary of Experimental Error Analysis. In Table II the various sources of error are listed with the magnitude of the errors in a hypothetical but realistic case. The variations in temperature, p_{H_2}, and pH are probably random and thus contribute principally to the standard error of $E^{\circ\prime}$ in a series of experiments. Errors in A_{red}/A_{ox} and the phenomenon of loss of chromophore from the protein are systematic in nature and are the chief sources of experimental uncertainty in $E^{\circ\prime}$.

Other Sources of Systematic Error in $E^{\circ\prime}$. Because the results of these studies have not been extrapolated to standard states, and because the appropriate activity coefficients are not known, the potentials reported here are not *standard* potentials. Rather they are practical values measured under conditions thought likely to be of physiological importance. Specific effects of ionic solutes were not investigated beyond the observations that there is no appreciable effect of buffer concentration within the limits of 0.05 and 0.2 M.

We have assumed that the absorbance properties of centers in proteins possessing two centers are equivalent. This assumption is borne out by both reductive (Evans et al., 1968) and oxidative titration (Eisenstein and Wang, 1969) studies on *C. pasteurianum* ferredoxin and is presumed to hold for the other eight iron, two center proteins.

Microscopic Potentials. Eisenstein and Wang (1969) have analyzed the case of a redox carrier containing two independent and equivalent single-electron sites, and in analogy to the case of the ionization of a dicarboxylic acid, have shown that the apparent redox potentials must differ by the statistical factor of 36 mV at 25 °C. They applied this analysis to data they obtained with equilibrium mixtures of *C. pasteurianum* ferredoxin and methylviologen. Aside from the fact that the $E^{\circ\prime}$ they assumed for the viologen is apparently incorrect (see below), note that their analysis makes rather strong demands on the accuracy of the data. For example, in Table III we show that there is little difference for the fraction ferredoxin reduced at various solution potentials, calculated for the statistical model and for the case where all centers have the same microscopic $E^{\circ\prime}$. We do not dispute the correctness of their analysis but are doubtful of its applicability to either our or their data. Our values of

Table III: Comparison of a[a] Values for Various Types of Oxidation–Reduction Models for Two-Center Ferredoxins.

$E°$(obsd) (mV)	a(obsd)[b]	Model[c,f]	Two Sites at Intermediate Potential[d]	A 425 Difference[e,f]
−320	0.0764	0.0734	0.0808	0.005
−342	0.166	0.164	0.171	0.005
−355	0.247	0.246	0.255	0.006
−366	0.335	0.340	0.345	0.003
−375	0.424	0.429	0.427	0.001
−385	0.529	0.529	0.524	0.003
−393	0.604	0.605	0.600	0.003
−398	0.661	0.658	0.646	0.008
−404	0.718	0.706	0.698	0.005

[a] $a = (a_1 + a_2)/2$ where a_1 is the fraction of one of the two centers reduced and a_2 is the fraction of the second center which is reduced. [b] Of Eisenstein and Wang (1969). Data collected by Eisenstein and Wang[f] for the potentials observed in the first column using 0.048 mM *C. pasteurianum* ferredoxin and 0.64 mM methylviologen. [c] a values calculated by Eisenstein and Wang for two sites having potentials of −367 and −398 mV. [d] a values calculated for model with two centers both with a potential of −382.5 mV. [e] Between the two model systems. [f] Under the conditions employed by Eisenstein and Wang (Eisenstein and Wang, 1969; Eisenstein, 1968), $da/d A_{425} = -1.48$, if one assumes that the absorbance at 425 nm due to the methylviologen can be determined precisely. Any uncertainty in A_{425} due to the methylviologen (such as possible dimerization or dye–protein interaction) could lead to further difficulties in choosing between the two model systems.

$E°'$ are the observed potentials when $[Fd_{red}]/[Fd_{ox}] = 1$, and in cases where two centers are present are the average of the microscopic potentials. Recently, Packer et al. (1975) have reported that the two clusters in both *C. acidi-urici* and *C. pasteurianum* ferredoxins are distinguishable, based on differences in ring carbon NMR shifts in the aromatic residues in the molecule. They find that the centers appear to have potentials differing by about 10 mV. As we showed above, our data would not allow this distinction to be made. Mathews et al. (1974) have proposed that the complexity of the EPR near $g = 2$ for reduced eight-iron ferredoxins is due to magnetic interactions between the centers. However, the energy range for the proposed interactions is below that observable in potentiometric experiments. Finally, Packer et al. estimate that the average potentials of *C. acidi-urici* and *C. pasteurianum* ferredoxins differ by 47 ± 10 mV in the presence of methylviologen. Our data confirm this estimate.

pH Dependence of $E°'$. The literature presents a varying picture of dependence of ferredoxin potentials on pH. Some investigators have found virtually no pH dependence (Tagawa and Arnon, 1968), whereas others have reported as much as 60 mV change/pH unit (Sobel and Lovenberg, 1966). These large changes can result from using incorrect values of n in Nernst's equation, as Eisenstein points out (1968). Although we have found a small pH dependence for all of the investigated ferredoxins, there were no cases which approached the degree of pH dependence expected if a free proton were obligatorily involved in the oxidation-reduction mechanism. The variable negative shift of $E°$ as the pH increases may be attributable to the deprotonation of a group at variable distances from the electron accepting site.

Selection of Wavelength Employed in Following the Oxidation–Reduction Reaction. Although higher sensitivity could be obtained by following the reduction of the ferredoxin at the wavelength of maximal change in absorbance upon reduction (ΔA_{max} usually is at about 420 nm for both bacterial- and plant-type ferredoxins), it was decided that the reduction should be followed at the λ_{max} of the protein (ca. 400 nm for bacterial ferredoxin and 420 nm for spinach ferredoxin). This was done to minimize errors arising from variations in the spectrophotometric center wavelengths and band passes.

Purity of Hydrogenase. The hydrogenase fraction used in this study was of sufficient purity and activity that less than 1% of the total protein on a molar basis in the reaction mixture was hydrogenase or other contaminating proteins (assuming a molecular weight of 60 000 for hydrogenase and other possible protein contaminants in the hydrogenase fraction). The use of small molar proportions of high specific activity hydrogenase avoids the formation of significant amounts of complexes between the ferredoxins and low-molecular-weight contaminants present in crude hydrogenase fractions.

Midpoint Potential of Methylviologen. The redox potentials of a variety of viologen dyes were originally reported by Michaelis and Hill (1933) as determined by potentiometric titrations at high pH values. The −446-mV potential for methylviologen was reported to be both pH and concentration independent. Other investigators have determined the redox potentials of methyl- and benzylviologen polarographically (Mueller, 1940; Elofson and Edsberg, 1957) but have obtained conflicting values. In spite of the uncertainty in the midpoint potential of methylviologen, occasional attempts have been made to determine the redox potentials of various proteins by equilibrating them with partially reduced methylviologen. By knowing the $E°'$ value for the dye and spectrophotometrically determining the ratios of reduced to oxidized species, it is possible to calculate a midpoint potential for the iron–sulfur protein.

The discrepancy between the $E°'$ value obtained for *C. pasteurianum* ferredoxin by equilibration with methylviologen (Eisenstein and Wang, 1969) and values determined in our studies led us to investigate the redox potential of 0.048 mM *C. pasteurianum* ferredoxin in the presence of 0.64 mM methylviologen. These are the same concentrations used in the experiments of Eisenstein (1968) and Eisenstein and Wang (1969). While the $E°'$ determined in this experiment for the ferredoxin was in excellent agreement with the value taken from Table I, the $E°'$ for methylviologen was −0.47 V. Experiments were initiated to determine the potential of methylviologen in the absence and presence of varying amounts of *C. pasteurianum* ferredoxin (Figure 1). Clearly, methylviologen and ferredoxin form a complex. The $E°'$ for methylviologen obtained in the absence of ferredoxin by this technique (−0.46 V) differed from the value (−0.446 V) reported by Michaelis and Hill (1933), so an attempt was made to duplicate the experiment of Michaelis and Hill. The value of −445 mV we obtained was in excellent agreement with the value of Michaelis and Hill. The polarographic determination, which showed evidence for weak absorption processes at the electrode surface, does little to clarify the exact $E°'$ value but does help to explain the different values found by other investigators who did not consider the effects of weak absorption or dimerization.

The results of the cyclic voltammetry experiments agreed quite well with the results of the H_2-hydrogenase experiments (−0.462 V with hydrogenase and about −0.46 V as determined by cyclic voltammetry). The results of these experiments are reinforced by the findings by Schwartz

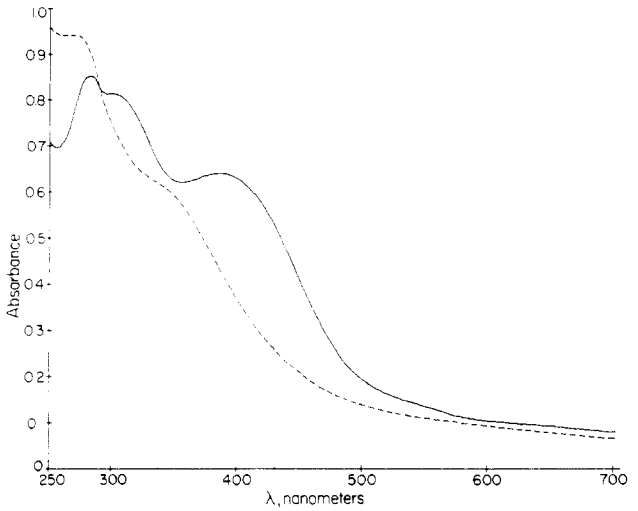

FIGURE 2: Electronic spectra of oxidized (—) and approximately 90% reduced (- - -) *Peptococcus aerogenes* ferredoxin, determined in 0.1 M potassium phosphate buffer (pH 7.9). The reduced spectrum was taken in the presence of 31 μg of *C. pasteurianum* hydrogenase and 1 atm of H_2. The volume in the cuvette was about 2.5 ml. The contribution of the hydrogenase to the reduced absorbance in the 250–325 nm region is less than 0.015; the contribution from 325–700 nm is essentially zero. The light path was 1 cm, and the samples were examined at room temperature (approximately 23 °C) in a Cary 14 spectrophotometer.

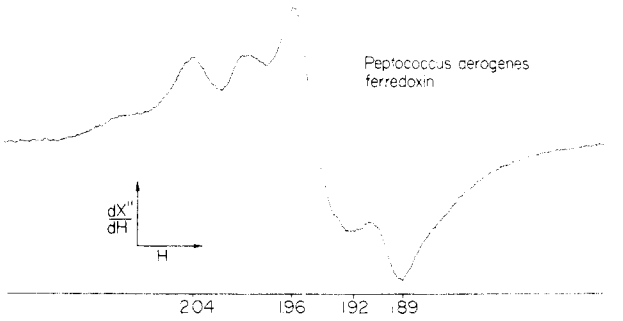

FIGURE 3: Electron paramagnetic resonance spectrum of approximately 90% reduced *Peptococcus aerogenes* ferredoxin. The sample was approximately 35 μM in ferredoxin and 0.1 M in potassium phosphate (pH 7.9). The protein was reduced by the presence of 31 μg of *C. pasteurianum* hydrogenase and 1 atm of H_2, and the total volume was 0.5 ml. Conditions of EPR spectroscopy: microwave frequency, 9.2 GHz; microwave power, 3 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; magnetic field sweep rate, 400 G min^{-1}; time constant, 0.25 s; sample temperature, 13 K. The abscissa is linear with field (several g values are given); the ordinate is an arbitrary function of the first derivative of the microwave absorption. The EPR spectrum of a similar sample lacking hydrogenase, when reduced with excess sodium dithionite, was essentially the same. The EPR spectrum of an oxidized sample of *P. aerogenes* ferredoxin shows a small $g = 2$ signal characteristic of all oxidized bacterial ferredoxins.

(1961) who found not only an $E°'$ value of −0.48 V for ethylviologen (30 mV more negative than the value found by Michaelis and Hill) with polarographic techniques, whose absorption errors were negligible, but also the same type of concentration dependence for ethylviologen as here reported for methylviologen with cyclic voltammetry. In addition, if the data of Eisenstein and Wang are recalculated with $E°'$ for methylviologen equal to −471 mV, the resulting $E°$ value for *C. pasteurianum* ferredoxin then is in excellent agreement with our value determined both in the presence and absence of methylviologen.

The results of these studies indicate that the redox potential of methylviologen at pH 7.4 is approximately −0.46 V. A real difference seemingly exists between the value as determined by potentiometric titration at alkaline pH's and the value obtained here near pH 7 by cyclic voltammetry and by enzymatic techniques.

Oxidation–Reduction Properties of Chromatium vinosum Ferredoxin. The unusually low oxidation–reduction potential of *Chromatium vinosum* ferredoxin ($E°'$ (pH 8.0) = −482 mV) as well as the unusually high A_{red}/A_{ox} value suggests that this protein possesses physical properties either in the electron-accepting site or in the protein moiety surrounding the iron–sulfur center, which distinguishes it from the other bacterial ferredoxins which we have investigated. Electron paramagnetic resonance spectra of this protein reduced by both the H_2/H^+ couple described in the text at pH 9.5 and 1 atm of H_2 ($E_{H_2}° = -560$ mV) and by a pyruvate dehydrogenase extract from *Bacillus polymyxa*[2] ($E° = -700$ mV) were identical, suggesting that the oxidation–reduction potential reported here is the only biologically relevant reaction, i.e., that no further reduction takes place, at least at potentials greater than −700 mV.

Oxidation–Reduction Properties of Peptococcus aerogenes Ferredoxin. Recently Adman et al. (1973) have reported the x-ray analysis of *P. aerogenes* ferredoxin to a 2.8-Å resolution. Since this protein is the only bacterial ferredoxin for which crystallographic data are presently available, its oxidation–reduction properties may be of special interest. The potentiometric data collected on this protein show great similarity to the data collected on the clostridial ferredoxins. Both the $E°$ (pH 7.0) and the $dE°/dpH$ values determined for *P. aerogenes* ferredoxin fall within the range of the clostridial values.

Presented in Figure 2 are the electronic spectra of both oxidized and reduced *P. aerogenes* ferredoxin; and in Figure 3 is presented the electron paramagnetic resonance spectrum of the reduced protein; these have not been published previously. These spectra as well as the oxidation–reduction properties of this ferredoxin add to the many other similarities of the clostridial and *P. aerogenes* ferredoxins. The common assumption that this group of ferredoxins have essentially identical iron–sulfur centers seems likely to be borne out by future structure studies.

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