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Characteristics of *Clostridium pasteurianum* Ferredoxin in Oxidation–Reduction Reactions*

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ABSTRACT: Studies of some characteristics of pure *Clostridium pasteurianum* ferredoxin pertinent to its role in oxidation–reduction reactions were performed.

It was found that reduced ferredoxin when treated with mercurials liberates two more atoms of iron(II) per mole than does oxidized ferredoxin; that there are no detectable free sulfhydryl groups in either species; that two electrons are transferred per mole of ferredoxin undergoing oxidation or reduction in several different reactions; and that the oxidation–reduction potential of ferredoxin varies systematically with pH.

Early studies of pyruvate oxidation and of hydrogen evolution in anaerobic organisms suggested the existence of a low potential electron carrier (Wolfe and O'Kane, 1953; Peck and Gest, 1957). Recently, Mortenson and his associates (1962) successfully isolated such a carrier which they named ferredoxin. It was found to be a low molecular weight protein containing nonheme iron, and it was demonstrated to be a necessary component in the phosphoroclastic cleavage of pyruvate by *Clostridium pasteurianum* (Mortenson *et al.*, 1963). Valentine and Wolfe (1963) implicated ferredoxin as a necessary cofactor in a wide variety of reactions occurring in many different anaerobes.

In 1962 Tagawa and Arnon noted the similarity between bacterial ferredoxin and the soluble nonheme iron protein cofactor involved in the photosynthetic reduction of pyridine nucleotides by choroplasts which had been described by San Pietro and Lang (1958). Although the chemical composition of clostridial ferredoxin has been studied (Lovenberg *et al.*, 1963), quantitative aspects of electron transport mediated by the protein have not been resolved. The data to be presented in this communication suggest that: iron in *C. pasteurianum* ferredoxin undergoes reversible oxidation and reduction concomitantly with that of the protein itself; that reduction of the protein is not associated with the appearance of free sulfhydryl groups; that the oxidation–reduction potential of ferredoxin is a function of pH; and that ferredoxin functions as a two-electron carrier.

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Experimental Section

Materials

Clostridium pasteurianum was grown in a medium containing ammonium sulfate as described previously (Lovenberg *et al.*, 1963).

Hydrogenase. A DEAE-cellulose treated extract obtained from *C. pasteurianum* after the cells had been sonically disrupted for 5 min in a Biosonik 20 kc sonic oscillator at 0° (Buchanan *et al.*, 1963) was used as a source of hydrogenase. The extract contained approximately 5 mg of protein/ml. Its activity was demonstrated by the reduction of methyl viologen by hydrogen (Peck and Gest, 1956).

Ferredoxin. Pure ferredoxin was prepared as described (Mortenson, 1964). Purity was ascertained by measurement of the A_{390}/A_{280} ratio on a Beckman DU spectrophotometer. All ferredoxin used in these studies had a ratio greater than 0.79. Even minimal deterioration of the protein was found to markedly alter experimental results. A molar extinction coefficient (390 mμ) of 3.0×10^4 for pure ferredoxin was used. This value was based on the molecular weight determined by sedimentation equilibrium studies, the extinction coefficient per milligram of protein as determined by the phenol protein assay (Lowry *et al.*, 1951), and the observation that the value for dry weight of pure ferredoxin was only 70% of that measured in the colorimetric reaction (Lovenberg *et al.*, 1963).

Ferredoxin that has been maximally reduced by dithionite has an absorbancy at 415 mμ equal to 46% that of the oxidized protein. This value was used in all calculations concerning per cent reduction in the experiments to be described. Reduction by dithionite was completely reversible.

Ferredoxin–Triphosphopyridine Nucleotide[1] *Reductase.* This enzyme was obtained from commercial spinach and purified through the second ammonium sulfate fractionation step by the procedure of Shin *et al.* (1963).

Chloroplasts. Active chloroplasts were obtained by a modification of the method of Whatley (Whatley *et al.*, 1959). Commercially obtained spinach was depetioled and then ground with cold sand for 2 min in two volumes of 0.4 M sucrose and 0.05 M Tris–HCl, pH 7.8. The slurry was filtered through double layer cheesecloth and centrifuged as previously described. Intact chloroplasts were used. Chlorophyll was assayed by the method of Arnon (1949).

Chemical Reagents. [1-^{14}C]Iodoacetic acid was obtained from New England Nuclear Corp. and recrystallized with carrier iodoacetic acid from chloroform. Glucose oxidase (fungal) was obtained from Calbiochem. All other reagents were obtained commercially.

Methods

Iron Determinations. The proportions of ferrous (II) and of ferric (III) iron were determined under anaerobic conditions using both oxidized and reduced ferredoxin. Such determinations were made after the iron had been released from ferredoxin by mercurical. Reduction of the protein was accomplished with illuminated chloroplasts (Fry *et al.*, 1963). Ferredoxin (0.1–1 μmole) in a reaction mixture containing 35 μmoles of Veronal buffer (pH 7.0 in most experiments) and 10 mg of glucose in a final volume of 3 ml was incubated in stoppered cuvets for 20 min, during which time the solution was bubbled with prepurified nitrogen. Subsequently, fresh chloroplasts containing a total of 50–100 μg of chlorophyll were added to the cuvet by injection through the serum cap stopper. Glucose oxidase, 0.5 mg, was added in a similar manner. The chloroplasts were added after the initial incubation because it was found that bubbling led to deterioration of their activity. Glucose and glucose oxidase were used to trap the oxygen generated and hence prevent the reoxidation of ferredoxin. After similar additions had been made to a blank, differing only by the absence of ferredoxin, the optical density at 415 mμ was determined. The cuvet was then exposed to light from a 150-w incandescent bulb filtered through 10 cm. of water (for the purpose of absorbing heat) for 8–10 min. OD_{415} was again promptly determined, and from its change the per cent reduction of ferredoxin could be calculated.

After reduction of ferredoxin had been achieved in one sample both that sample and another which had been kept in the dark and contained, therefore, oxidized anaerobic ferredoxin were treated similarly. Iron was released from the protein by treating the sample with mercurial, and an assay of the valence state of free iron appearing was performed. Mercuric acetate solution (10^{-1} M) which had been prepared with deaerated water was injected into both solutions in a quantity which had been ascertained to be minimal and yet sufficient to bleach the visible spectrum of the ferredoxin. Occasionally, *p*-chloromercurisulfonate was used instead. Such a procedure leads to the liberation of iron from the protein (Lovenberg *et al.*, 1963). A 1-ml aliquot of each solution was added to 1 ml of glacial acetic acid, and the standard *o*-phenanthroline assay was performed by adding 1 ml of 0.3% *o*-phenanthroline (Harvey *et al.*, 1955). The proportion of iron(II) was determined by reading OD_{512} after 10 min, and comparing it to OD_{512} obtained after the addition of 0.1 ml of 28% hydroxylamine, a reagent which promptly reduced iron(III) to iron(II).

Several aspects of the above deserve special mention. First, Veronal was chosen as a buffer because studies with standard iron solutions incubated briefly with very low concentrations of phosphate, borate, or Tris buffers over a wide pH range showed varied and marked interference with the development of total color at the end of the assay and alteration in the proportion of initial color to color after the addition of hydroxylamine. These effects were exaggerated by alkalinity of the original buffer solution, but occurred to some degree whenever the pH was greater than 6. Anthranilic acid was utilized to detect iron(III) in the presence of these buffers (Dinsel and Sweet, 1963), and it was found that standard solutions of iron(II) were not being converted to free iron(III) to any appreciable extent. Interference apparently reflected binding of iron by the buffer itself. Other incubations with low concentrations of NaOH, without buffer, demonstrated interference also, suggesting that the iron(II) was precipitated under such circumstances.

Second, as was pointed out in early literature concerning the *o*-phenanthroline assay (Fortune and Mellon, 1938), mercury interferes with the assay even at low concentration. Recovery experiments demonstrated that, if the amount of mercury added to the ferredoxin sample was sufficient but not in excess of that required to bleach the protein's visible spectrum, the determination of standard iron added to the final mixture was accurate. Excess mercury led to erroneous results even when turbidity had been removed by centrifugation. Under these circumstances not only the value for total iron but also that for the proportion of iron(II) to total iron differed from values obtained with the untreated standard solutions.

Third, a possible source of error produced by excess reductant, as pointed out by Massey (1957), was avoided by using the chloroplast system. Recovery experiments indicated that iron(III) added to a sample of ferredoxin previously reduced with light was not reduced to iron(II) rapidly enough to interfere with the assay. Since iodoacetate incorporation (see below) was the same in oxidized and in reduced ferredoxin, it seems improbable that an increased amount of reducing power attributable to sulfhydryl groups would be present in reduced *vs.* oxidized ferredoxin. Comparisons were made only between samples containing the same amount of pro-

[1] Abbreviations used in this work: TPN^+, oxidized triphosphopyridine nucleotide; TPNH, reduced TPN.

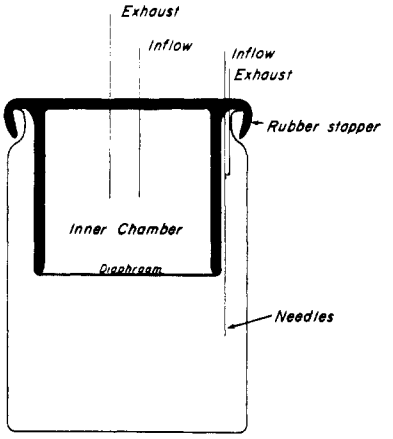

FIGURE 1: Anaerobic double-chambered cuvet.

tein and treated with the same amount of mercurial.

Fourth, it was noted that the proportion of initial OD_{512} to OD_{512} following the addition of hydroxylamine after iron had been liberated from ferredoxin was pH dependent. Such a phenomenon did not occur with standard iron(II) or iron(III) solutions. In the studies on the protein then, all samples compared (oxidized *vs.* reduced ferredoxin) were maintained at the same pH with Veronal buffer.

Finally, it was noted that the presence of glucose, glucose oxidase, and oxygen inhibited the degree of color generated by a standard iron(II) solution. Iron(III) was not detected by the anthranilic acid assay. The possibility of conversion of iron(II) to iron(III) had been suggested by the occurrence of H_2O_2 as a product in the glucose oxidase reaction. Glucuronic acid itself (the other product) did not interfere with the iron assay, and no combination of the reactants and enzyme other than that cited led to interference. Since a small amount of oxygen is generated by the chloroplasts in the reduced ferredoxin samples and none is generated in the anaerobic oxidized ferredoxin samples, we would anticipate a tendency to detect more apparent iron(III) in the reduced samples. This will be considered below.

Determination of Free Sulfhydryl Groups in Oxidized and in Reduced Ferredoxin. The possible presence of free sulfhydryl groups in ferredoxin was examined by measuring incorporation of [^{14}C]iodoacetate into the protein. The reaction was performed in anaerobic cuvets containing 0.1 M Tris–HCl, pH 8.6, 4.0 M urea, and 0.1 mM ferredoxin after purging with either hydrogen or nitrogen for 30 min. Subsequently, 10 μl of hydrogenase was added anaerobically. The degree of reduction of ferredoxin in each sample was measured spectrophotometrically. No reduction occurred in the absence of hydrogen. After maximum reduction had been achieved (90–100%) 10 μmoles of [^{14}C]iodoacetate (14,000 cpm/μmole) was added anaerobically and the reaction mixture was incubated for 1 hr at room temperature. The anaerobic oxidized ferredoxin sample (N_2 atmosphere) was treated similarly. Subsequently, iodoacetate which had not reacted with the protein was separated by chromatography on Sephadex G-25. The radioactivity in the protein fraction of each sample was measured in a liquid scintillation counter. From this, the amount of iodoacetate incorporated per mole of ferredoxin was calculated.

Oxidation–Reduction Potential. The oxidation–reduction potential of ferredoxin was determined (Tagawa and Arnon, 1962) by measuring the degree of reduction of ferredoxin in the presence of hydrogen and hydrogenase. The per cent reduction of the protein was determined spectrophotometrically by following OD_{415}. Since

$$E_h = E_0' + \frac{RT}{nF} \ln \frac{\text{oxidized}}{\text{reduced}}$$

(Clark, 1960) and since at equilibrium

$$E_h = \frac{(H^+)}{(H_2)^{1/2}}$$

E_0' could be calculated at any given pH after the barometric pressure and vapor pressure of water were used to calculate the partial pressure of H_2. The number of equivalents of electrons transferred per mole, n, was assumed to be two based on results to be reported below. For purposes of calculation concentrations were used without correction for possible discrepancies between concentration and activity. The reactions were carried out at 25° in stoppered cuvets which were bubbled with hydrogen for 1 hr and contained 0.68 μmole of ferredoxin and 300 μmoles of Tris–HCl or phosphate buffer at a specific pH, in a final volume of 3 ml. Hydrogenase (20 μl of the extract) was added to initiate the reaction and equilibrium was apparently attained within 10 min.

Determination of n. The number of equivalents of electrons transferred per mole of ferredoxin undergoing oxidation or reduction was examined utilizing several different methods.

(1) By measuring optical density at two different wavelengths for which extinction coefficients for oxidized and reduced members of two interacting species are known, the per cent reduction of each at equilibrium can be determined (Stotz *et al.*, 1938). TPNH and ferredoxin were incubated in anaerobic cuvets (Figure 1) in a final volume of 3 ml, 0.1 M with respect to Tris–HCl, pH 7.0, and deaerated with prepurified nitrogen for 1 hr. Ferredoxin–TPN^+ reductase (10 μl) which had been deaerated in the inner chamber was introduced to initiate the reaction by perforating the diaphragm separating the two chambers. The quantity of ferredoxin reduced and of TPNH oxidized was measured spectrophotometrically after molar extinction coefficients at 340 and 415 mμ had been determined. For oxidized ferredoxin the values were 2.88 and 2.76 $\times$ 10^4, respectively. Corresponding values for ferredoxin which had been reduced by hydrogen and hydrogenase were 2.77 and 1.21 $\times$ 10^4. The value used for TPNH at 340 mμ was 6.2 $\times$ 10^3 (Horecker and Kornberg, 1948).

TABLE I: The Oxidation State of Iron Released from Oxidized and from Reduced Ferredoxin.[a]

Expt	Sample	OD_{415} (initial)	OD_{415} (after redn by light)	Reduction (%)	OD_{512} (initial)	OD_{512} (after NH_2OH)	Ferrous Iron (%)	Increase in μatoms of Iron(II)/ μmole of Ferredoxin Reduced
1	Reduced	0.730	0.350	85	0.410	0.570	72	1.5
	Oxidized	0.790	0.780	0	0.305	0.560	54	
2	Reduced	1.465	0.765	90	0.820	1.000	82	1.8
	Oxidized	1.480	1.485	0	0.590	0.995	59	
3	Reduced	0.485	0.235	93	0.240	0.320	75	1.8
	Oxidized	0.485	0.470	0	0.160	0.310	51	
4	Reduced	1.060	0.505	98	0.530	0.710	74	1.8
	Oxidized	1.040	1.060	0	0.338	0.690	48	

[a] See text for experimental details.

Neither TPNH nor TPN^+ have appreciable absorbancy at 415 mμ. Optical density was measured on a Beckman DU spectrophotometer equipped with a Gilford optical density converter. This instrument had been demonstrated previously to provide excellent precision and linear responses up to optical density readings of 2.5 at these wavelengths.

(2) Utilizing similar spectrophotometric measurements the reverse reaction was studied stoichiometrically. Ferredoxin was reduced in the presence of light and chloroplasts (containing approximately 50 μg of chlorophyll) which had been heated previously for 5 min at 55° in order to inhibit their capacity to generate oxygen. 2,6-Dichlorophenolindophenol (0.2 μmole) and ascorbic acid (30 μmoles) were used as the electron source (Whatley *et al.*, 1963). This mixture was incubated in Thunberg cuvets (final volume, 3 ml) in 0.1 M Tris–HCl, pH 7.0, and made anaerobic with prepurified nitrogen. TPN,[1] present in the side arm, was then added to the reaction mixture and the amounts of TPN^+ reduced and of ferredoxin oxidized were measured spectrophotometrically.

It was felt that the major systematic error in these two approaches to the determination of n would be attributable to trace amounts of oxygen. Since ferredoxin is autoxidizable, such an error would tend to produce falsely high values for n in the first case and falsely low ones in the second. Control experiments utilizing the reduction of methylene blue (0.1 μmole reduced) and of flavin–adenine dinucleotide (0.089 μmole reduced) by TPNH (0.093 μmole oxidized) yielded the anticipated results for the stoichiometry of electron transport with each pair. In other experiments it was found that TPNH was not nonspecifically oxidized by the disulfides in bovine pancreatic ribonuclease or oxidized glutathione under the same conditions even in the presence of ferredoxin–TPN reductase.

(3) The stoichiometry of the following reaction was studied using a Warburg respirometer.

$$H_2 + Fd_{oxidized} \rightarrow Fd_{reduced} + 2H^+$$

where Fd represents ferredoxin. Ferredoxin (1.5–3.1 μmoles) in 0.1 M Tris–HCl, pH 7.0, was incubated in a hydrogen atmosphere in the main body of the reaction vessel. Hydrogenase (100 μl of the extract, *ca.* 5 mg/ml of protein) was in the side arm. After equilibration the reaction was initiated by adding the enzyme to the main body of the flask. Per cent reduction of ferredoxin was determined spectrophotometrically in separate experiments under similar conditions, and the uptake of hydrogen was measured manometrically.

Control experiments using the same apparatus and conditions were performed using indigotrisulfonate as the electron acceptor and hydrogen as the electron donor. The anticipated stoichiometry was observed (Sullivan *et al.*, 1923). H_2 (4.4 μmoles) was taken up and 4.0 μmoles of dye was reduced.

(4) Potentiometric oxidation–reduction titrations were performed at 25° in a 5-ml titration vessel enclosed in a water jacket. A plastic head contained inlets for a platinum electrode, a saturated calomel electrode, a buret tip, and a polyethylene gas inflow tube. A removable exhaust was provided. The solution was constantly mixed by means of a magnetic stirrer. A type TTT1C Radiometer galvanometer system was used. All potentials were corrected to E_h values by adding the standard values for the calomel electrode at the prevailing temperature to the observed potential. A reservoir buret, calibrated to 0.01 ml, was used for the addition of titrant after its contents had been purged with prepurified nitrogen for several hours.

The titration vessel contained ferredoxin (0.3–1.0 μmole of different preparations) in 0.1 M Tris–HCl in a volume of 3 ml. After deaeration of the vessel for 1 hr, sodium dithionite, prepared essentially as described by Harbury (1957), was added in a quantity just sufficient to completely reduce the ferredoxin. In some experiments solid dithionite was added directly to the reaction mix-

TABLE II: The Stoichiometry of Ferredoxin Reduction by TPNH.[a]

Expt	Initial OD	OD (after addition of ferredoxin–TPN reductase)	TPNH Oxidized[b] (mμmoles)	Ferredoxin Reduced[b] (mμmoles)	n[b]
1	2.348 at 340 mμ 1.392 at 415 mμ	2.262 1.261	30.0	26.2	2.3
2	2.452 at 340 mμ 1.390 at 415 mμ	2.368 1.258	28.5	26.1	2.2

[a] The reaction mixture consisted of 2 μmoles of TPNH, 0.16 μmole of ferredoxin, 300 μmoles of Tris–HCl, pH 7.0, in a final volume of 3 ml in anaerobic cuvets (Figure 1). The reaction was initiated after purging with prepurified N_2 for 1 hr by anaerobically introducing 10 μl of ferredoxin–TPN^+ reductase (*ca.* 5 mg/ml). To facilitate the determination of OD_{340}, the blank contained 0.75 μmole of TPNH. Extinction coefficients required in the calculations are discussed in the text. The (change in OD_{340})/(change in OD_{415}) when ferredoxin is reduced by dithionite or hydrogen and hydrogenase was found to be 0.186. Using this fraction the change in OD_{340} due to TPN^+ reduction and hence mμ-moles of TPN^+ undergoing reduction was calculated. [b] See text for method of calculation.

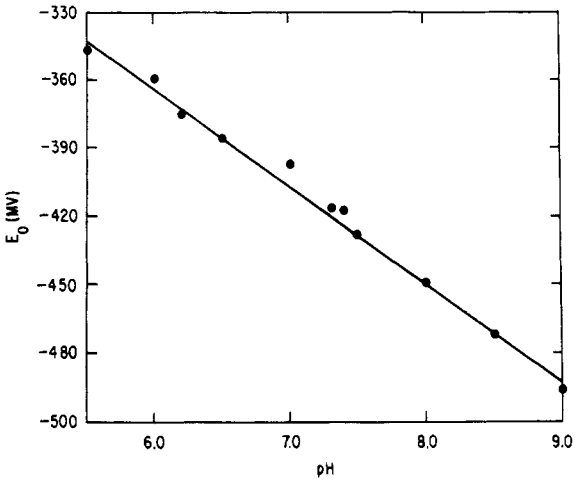

FIGURE 2: Variation of the oxidation–reduction potential of ferredoxin with pH. The experimental procedure is discussed in the text.

ture in 0.025-mg amounts to a total of approximately 0.15 mg. Subsequently, after a stable potential had been achieved, oxidative titration was performed by the addition of deoxygenated dinitrophenolindophenol (10^{-3} M in most experiments). The number of electrons transferred per mole of ferredoxin undergoing oxidation was determined from the slope of the potentiometric titration curve (Clark, 1960).

Results

Iron Determination. Data concerning the proportion of observed iron(II) released from oxidized and reduced ferredoxin are presented in Table I. It can be seen that an apparent change in the oxidation state from III to II of 1.7 iron atoms released from ferredoxin is associated with reduction of each mole of the protein. As discussed above, there is a tendency to observe relatively more iron(III) in the reduced samples because of the generation of some oxygen by chloroplasts and the presence of glucose and glucose oxidase in the system. Thus, these results are consistent with the nearest integral value, 2, for the increase in the observed number of iron atoms released in the ferrous state per mole of ferredoxin undergoing reduction.

Free Sulfhydryl Groups in Oxidized and in Reduced Ferredoxin. It has been reported previously (Lovenberg *et al.*, 1963) that very little [^{14}C]iodoacetate is incorporated into oxidized ferredoxin. However, reduction of ferredoxin with mercaptoethanol in a nitrogen atmosphere leads to subsequent alkylation of all the cysteine residues following incubation with iodoacetate. In this study when ferredoxin was incubated with [^{14}C]-iodoacetate in the presence of hydrogenase and either nitrogen (oxidized ferredoxin) or hydrogen (reduced ferredoxin), less than 0.1 mole of iodoacetate was incorporated per mole of protein. Such observations suggest that no sulfhydryl groups are exposed during biological reduction of the protein.

Determination of the Oxidation–Reduction Potential of Ferredoxin. By utilizing the hydrogen and hydrogenase system the oxidation–reduction potential for ferredoxin as a function of pH was obtained. These data are depicted graphically in Figure 2. Multiple points at the same pH indicate determinations performed in the presence of different buffers. When calculations are done with a value of n equal to one, our results show good agreement with those calculated by Tagawa and Arnon on the same basis over the narrow pH range which they reported (Tagawa and Arnon, 1962). The curve presented in the figure is, however, the result of calculations based on a value of $n = 2$, selected on the basis of data to follow. It can be seen that E_0 varies with pH, suggesting that there is a change in the "active reductant" (or oxidant) contributed per mole of ferre-

doxin related to hydrogen ion concentration (Clark, 1960).

Determination of n (the number of equivalents of electrons transferred per mole of ferredoxin oxidized or reduced). Results from experiments in which ferredoxin was reduced by the addition of TPNH are presented in Table II. Calculations of the value for n were done as follows. Since the final volume was 3 ml, the number of μmoles of TPNH oxidized = $3(\Delta OD_{340}$ due to TPNH oxidation)/6.2, and since the OD_{415} of reduced ferredoxin = $0.46OD_{415}$ of oxidized ferredoxin, the per cent reduction of ferredoxin was obtained from $0.54(\Delta OD_{415})/OD_{415,\ initial}$. Since TPNH donates two electrons in the course of its oxidation, n for ferredoxin = 2(μmoles of TPNH oxidized)/[(μmoles of ferredoxin) % reduction].

The second approach to the determination of n utilized ferredoxin reduced by light and chloroplasts to which TPN^+ was subsequently added anaerobically. Again the stoichiometry was observed spectrophotometrically by measuring the optical density at 415 and at 340 mμ. Data derived from experiments employing this approach are presented in Table III. These first two means of determining the value of n lead to similar results, of particular interest because the presence of oxygen in trace amounts, probably constituting the most significant systematic error, would affect the results in the two sets of experiments in contrary directions. In both cases, however, values obtained for n were approximately two.

A third approach to the determination of n entailed the measurement of hydrogen uptake per mole of ferredoxin reduced when hydrogenase was added to anaerobic oxidized ferredoxin contained in a Warburg vessel. Data derived from such studies are presented in Table IV. Virtually identical results were obtained from different preparations of ferredoxin and with different Warburg flasks and manometers independently calibrated. Again, the value of n obtained was approximately two.

Finally, n was determined from the slope of the mid region of potentiometric oxidation–reduction titration curves, examples of which are presented in Figure 3. As noted by others, potentials in the mid regions of the curves were stable within minutes, while those in the peripheral portions were stable only after 30 min to 1 hr (Harbury, 1957). The slopes of all curves obtained were virtually identical and consistent with a value of $n = 2$.

TABLE III: The Stoichiometry of TPN^+ Reduction by Ferredoxin.

Expt	pH	Initial OD (415 mμ)	Initial OD (340 mμ)	OD (after Exposure to light) (415 mμ)	OD (after Exposure to light) (340 mμ)	Ferredoxin Present[a] (μmole)	Redn of Ferredoxin by Light[b] (%)	OD (after Equilibration) (415 mμ)	OD (after Equilibration) (340 mμ)	Redn of Ferredoxin at Equilibrium[b] (%)	TPNH Formed[c] (μmole)	Ferredoxin Oxidized by TPN^+ (μmole)	n
1	8.0	1.042	1.143	0.560	1.061	0.114	86	1.016	1.375	4	0.110	0.095	2.3
2	7.0	1.340	1.450	0.710	1.215	0.146	87	1.255	1.650	11	0.117	0.112	2.1
3	7.0	1.142	1.215	0.740	1.205	0.124	65	1.110	1.431	7	0.076	0.072	2.1

[a] Molar extinction coefficient (415 mμ) for ferredoxin, independently determined, = 2.76×10^4. [b] The change in absorbancy at 340 mμ due to oxidation of ferredoxin = 0.186 times the observed change in absorbancy at 415 mμ when TPN^+ is added to the reaction mixture. This quantity was subtracted from the change in absorbancy at 340 mμ which was actually observed in order to obtain a value used in calculating the amount of TPNH formed. [c] Per cent reduction of ferredoxin = $(OD_{415,initial} - OD_{415,final})0.54/OD_{415,initial}$. This is based on the observation that OD_{415} decreases by 54% when ferredoxin is reduced with dithionite. Such reduction is completely reversible. All optical density readings have been corrected for the final volume of 3 ml.

Discussion

Some of the major bacterial reactions in which ferredoxin has been implicated as a participating cofactor (Valentine and Wolfe, 1963) include: oxidation of hypoxanthine, pyruvate, and α-ketoglutarate, and reduction of nitrite, pyridine nucleotides, and hydroxylamine by molecular hydrogen. In most of these reactions 2 equiv of electrons is transferred per mole of reduced or oxidized substrate. In some of them, artificial one-electron carriers (*e.g.*, the viologens) are less effective than ferredoxin as mediators (Tagawa and Arnon,

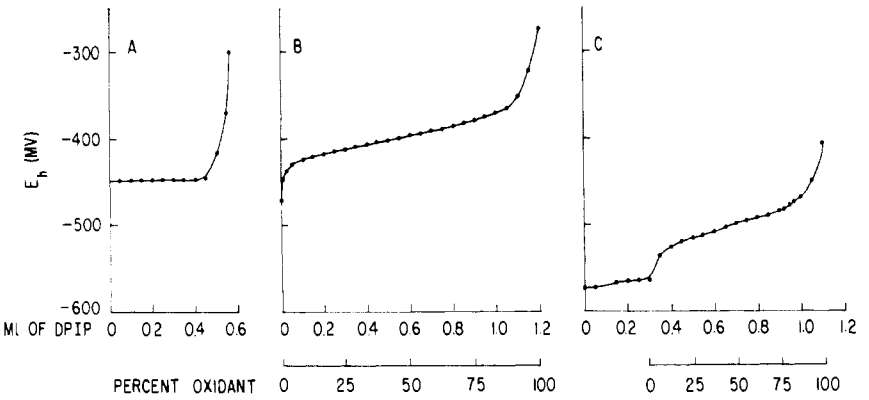

FIGURE. 3: Potentiometric oxidation–reduction titration of ferredoxin. (A) Oxidative titration of sodium dithionite in the absence of ferredoxin at pH 7.0. (B) Oxidative titration of reduced ferredoxin at pH 7.0. (C) Oxidative titration of reduced ferredoxin at pH 9.0. The initial reduction of ferredoxin was accomplished with sodium dithionite. See text for experimental procedure.

TABLE IV: The Stoichiometry of Ferredoxin Reduction by Hydrogen.[a]

Expt	Manometer Readings after Equilibration with H_2 with Respect to a Constant Reference Point (mm)		Subsequent to the Addition of Hydrogenase (mm)		Ferredoxin in the System (μmoles)	Ferredoxin Reduced[b] (μmoles)	pH	Flask Constant (k)[c]	H_2 taken from Gas Phase[c] (μmoles)	n[d]
	Thermobarometer	Reaction flask	Thermobarometer	Reaction flask						
1	2	33	5	−12	3.01	2.48	7.4	1.34	2.62	2.1
2	0	35	−1	−15	3.01	2.48	7.4	1.34	2.68	2.2
3	37	54	53	36	1.82	1.69	8.0	1.37	1.87	2.2
4	42	28	44	0	1.82	1.48	7.4	1.23	1.47	2.0

[a] Expt 1 and 2 were carried out at 25°. The value for the molar volume of H_2 at this temperature used in the calculations was 24.5 l. In experiments 3 and 4, carried out at 30°, the corresponding value was 25 l. Each reaction flask contained ferredoxin, 0.1 M Tris–HCl, and a final volume of 4 ml. The side arm contained hydrogenase (*ca.* 5 mg/ml), 0.1 ml, which was added to the main flask after equilibration with H_2 was complete, usually within 1 hr. [b] This was calculated from the per cent reduction of ferredoxin previously determined spectrophotometrically at the appropriate pH. At pH 7.4 the value is 82%, while at pH 8.0 it is 93%. [c] k = (μl Δvolume)/(mm Δpressure). It incorporates the Bunsen coefficient for H_2 at the appropriate temperature and the volume of fluid in the reaction flask. [d] n = 2(μmoles of H_2 taken up from gas phase)/(μmoles of ferredoxin reduced).

1962). The presence of a free radical form of ferredoxin has not been reported. In view of these considerations it was felt that the currently accepted value of $n = 1$ (Tagawa and Arnon, 1962) deserved further scrutiny. Our results, deriving from four independent methods, strongly suggest that the appropriate value for this parameter is $n = 2$.

Of interest also are the studies concerning the valence state of iron released from oxidized and from reduced ferredoxin. The change observed is consistent with a change from the ferric to ferrous state of two atoms of iron/mole of protein undergoing reduction. Although the value for the amount of iron(II) in oxidized ferredoxin differs from that reported by Bloomstrom *et al.* (1964), perhaps for the reasons discussed above, the fact that a difference in 2 atoms of iron/mole of reduced compared to oxidized ferredoxin was observed provides further support for the concept that the value of n is 2. This of course implies that the change in valence state of iron observed is a direct reflection of the number of electrons transferred, an as yet not established but plausible possibility.

The data concerning the effect of pH on the E_0 of ferredoxin are of interest in view of the physiological

role of the electron carrier and the relationship of its E_0 to the potential (E_h) of the standard hydrogen electrode. Systematic variation in E_0 at varying pH was noted in studies utilizing hydrogen and hydrogenase as well as in the potentiometric oxidation–reduction titrations. Agreement between the E_0 value at any given pH obtained by the two methods was quite good. E_0-(ferredoxin) can be seen to be progressively more positive with respect to the standard hydrogen electrode at higher pH values. For example, at pH 6, E_0(ferredoxin) = −360 mv, and E_h(standard hydrogen electrode) = −360 mv also. At pH 9, however, E_0(ferredoxin) = −496 mv, and E_h(standard hydrogen electrode) = −540 mv. This relationship would favor a larger proportion of ferredoxin being in the reduced state at the same partial pressure of hydrogen at alkaline pH in comparison with a more acid condition. Thus, the production of H^+ ion due to oxidation of hydrogen would be more prevalent when the organism could better "afford it" in terms of the degree of alkalinity present. Similarly, under acid conditions, this relationship would favor the formation of molecular hydrogen at the expense of reduced substrates and tend to decrease the H^+ concentration. Although the rates of these reactions are not uniquely determined by the relative oxidation–reduction potentials of the interacting species, the concentrations of such species would reflect the relative E_0 if equilibrium were to be attained. In view of the fact that hydrogen can escape from the system, probably as a function of the rate of its generation, the systematic variation of E_0(ferredoxin) may well provide a means of maintaining homeostasis with respect to H^+ ion.

Acknowledgment

The authors gratefully acknowledge helpful suggestions provided by Dr. Henry A. Harbury, Dr. Donald L. Keister, and Dr. Samuel Raeburn. We appreciate also the skillful technical assistance of Miss Eleanor Cavanaugh.

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