The Stoichiometry of Electron Transfer by Bacterial and Plant Ferredoxins

By M. C. W. EVANS, D. O. HALL, H. BOTHE AND F. R. WHATLEY

Department of Botany, King's College, University of London,
Half Moon Lane, London, S.E. 24

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1. The number of electrons carried by ferredoxins from spinach, the blue-green alga *Anacystis nidulans*, the anaerobic bacterium *Clostridium welchii* and the photosynthetic bacterium *Chromatium* was determined. 2. Ferredoxins were reduced by illuminated chloroplasts, and the stoichiometry of the reoxidation in the dark of the ferredoxins by NADP and benzyl viologen was measured. 3. Spinach and *A. nidulans* ferredoxins were found to be one-electron carriers, and *Cl. welchii* and *Chromatium* ferredoxins were two-electron carriers.

The number of electrons accepted by spinach ferredoxin on reduction by illuminated chloroplasts was determined by Whatley, Tagawa & Arnon (1963) and by Horio & San Pietro (1964) by measuring the stoichiometry of the reoxidation of the ferredoxin by NADP. Both groups found that spinach ferredoxin accepted one electron/molecule on reduction. Sobel & Lovenberg (1966) measured the number of electrons accepted by bacterial ferredoxin from *Clostridium pasteurianum* by the method of Whatley *et al.* (1963) and by several other methods. They found that clostridial ferredoxin accepted two electrons/molecule on reduction. Tagawa & Arnon (1968) redetermined the number of electrons accepted by clostridial ferredoxin by using the stoichiometry of ferredoxin reoxidation by NADP or benzyl viologen and obtained a value of one electron/ferredoxin.

During our work on the physical properties of ferredoxins we have determined the number of electrons accepted by two bacterial ferredoxins and two plant ferredoxins. The bacterial ferredoxins were from the photosynthetic bacterium *Chromatium* and from the non-photosynthetic anaerobe *Clostridium welchii*. The plant ferredoxins were from spinach and from the blue-green alga *Anacystis nidulans*. Using both of the methods described by Tagawa & Arnon (1968), we find that the bacterial ferredoxins accept two electrons/molecule on reduction, and that spinach and *Anacystis* ferredoxins each accept one electron/molecule. A preliminary account of this work has been published (Evans, Hall, Bothe & Whatley, 1968).

MATERIALS AND METHODS

Sources of ferredoxin. *Chromatium* strain D was grown in a medium based on medium S described by Lascelles (1956),

supplemented with 40 ml. of Pfennig's (1962) heavy-metals solution/l., and with the vitamins and sodium glutamate omitted. After harvesting, the cell paste was stored frozen until required. Cell paste of *Cl. welchii* was a gift from Mr W. Foster of the Wellcome Foundation, Beckenham, Kent. Spinach was purchased locally and stored frozen until required.

Preparation of ferredoxins. Ferredoxins from *Chromatium* and *Cl. welchii* were prepared by similar procedures. The cell paste was suspended in 0.05 M-potassium phosphate buffer, pH 7.3 (1:1, w/v). Batches (300 ml.) of the suspension were disrupted by ultrasonic oscillation for 5 min. with the maximum output of a Dawe Soniprobe. Acetone at -20° was then added to the crude extract (1 vol./vol. of buffer), and the mixture was kept at 0° for 30 min. After centrifugation for 30 min. at 18000g, the supernatant was passed through a column (10 cm. $\times$ 3 cm.) of DEAE-cellulose equilibrated with 0.02 M-potassium phosphate buffer, pH 7.3, to collect the ferredoxin. The same size of column was used for up to 2 kg. of cell paste. The column was then washed with 200–300 ml. of 0.26 M-NaCl in 0.01 M-potassium phosphate buffer, pH 7.3, and ferredoxin was eluted with 0.8 M-NaCl in 0.01 M-potassium phosphate buffer, pH 7.3. The eluate was then diluted with 3 vol. of water, and the ferredoxin was collected on a column (10 cm. $\times$ 2.5 cm.) of DEAE-cellulose. This column was then washed with 0.3 M-NaCl in 0.01 M-potassium phosphate, pH 7.3, until the ferredoxin had spread through half the column, and the ferredoxin was then eluted with 0.8 M-NaCl in 0.01 M-potassium phosphate buffer, pH 7.3. The eluate was diluted with 3 vol. of water and the ferredoxin was collected on a column (40 cm. $\times$ 2.5 cm.) of DEAE-cellulose. The ferredoxin was eluted from this column with 0.36 M-NaCl in 0.01 M-potassium phosphate, pH 7.3 (*Cl. welchii*), or 0.4 M-NaCl in 0.01 M-potassium phosphate, pH 7.3 (*Chromatium*). Fractions from the column with E_{390}/E_{280} ratio 0.65 or greater were collected, concentrated, freed of NaCl by dialysis and used in the experiments. The best preparations of *Chromatium* ferredoxin obtained by this procedure had E_{390}/E_{280} ratio 0.76 and of *Cl. welchii* ferredoxin 0.73. Further purification by $(\text{NH}_4)_2\text{SO}_4$ fractionation

(Mortenson, 1964) resulted in preparations with a ratio of 0.79 for *Chromatium* ferredoxin. Minor variations in purity, indicated by ratios between 0.6 and 0.75, did not affect the results obtained if freshly prepared ferredoxin was used.

Spinach ferredoxin was prepared as described by Tagawa & Arnon (1962) except that the tris-HCl buffer-NaCl mixtures used by them were replaced by appropriate concentrations of NaCl in 0.01 M-potassium phosphate buffer, pH 7.3.

The preparation of *A. nidulans* ferredoxin was essentially the same as for spinach ferredoxin (H. Bothe, unpublished work).

Extinction coefficients of ferredoxins. The millimolar extinction coefficient for spinach ferredoxin at 420 m μ , $E_{420}^{1\text{mm}}$, 9.7×10^3 , given by Tagawa & Arnon (1968), was used, as was their value of a 51% fall in E_{420} on complete reduction of spinach ferredoxin. *Anacystis* ferredoxin was assumed to have a similar extinction coefficient. The extinction coefficient of *Chromatium* ferredoxin at 385 m μ was determined by Sasaki & Matsubara (1967): $E_{385}^{1\text{mm}}$, 3.1×10^4 . On the basis of this value, we found the millimolar extinction coefficient at 415 m μ to be 2.86×10^4 . The maximum change in E_{415} observed on reduction of this ferredoxin in these experiments was 47%. The isolation of ferredoxin from *Cl. welchii* has not previously been reported. Sobel & Lovenberg (1966), however, reported the millimolar extinction coefficient of *Cl. pasteurianum* ferredoxin. Measurement of the extinction coefficient of *Cl. welchii* at 390 m μ based on the phenol method of protein determination (Rabinowitz & Pricer, 1962) indicated $E_{390}^{1\%}$, 31, compared with 33 for *Cl. pasteurianum* ferredoxin (Mortenson, 1964). Preliminary amino acid analysis showed that *Cl. welchii* ferredoxin has an amino acid composition similar to that of *Cl. pasteurianum* ferredoxin and other clostridial ferredoxins, indicating a similar molecular weight. The millimolar extinction coefficient at 415 m μ given by Sobel & Lovenberg (1966), $E_{415}^{1\text{mm}}$, 2.76×10^4 , for *Cl. pasteurianum* ferredoxin was therefore used in the experiments with *Cl. welchii* ferredoxin. The maximum change in E_{415} that we observed on reduction was 51%.

EXPERIMENTAL PROCEDURE

All the determinations were carried out by using anaerobic Thunberg cuvettes with a 1 cm. light-path. The cuvettes had a normal Thunberg top, and an additional small side arm was fused to the body of the tube between the cuvette and the ground-glass joint. The reaction mixture was placed in the cuvette except for the final electron acceptor, which was in the small side arm. A small amount of pyrogallol was placed in the top of the tube, and 0.5 ml. of 10% (w/v) Na_2CO_3 was added to it immediately before the tube was gassed. The tube was made anaerobic by evacuation followed by flushing with argon, this was repeated a number of times and the tube was finally filled with argon. The alkaline pyrogallol served to scrub any traces of oxygen from the atmosphere of the tube. Glucose and glucose oxidase were added to all reaction mixtures to remove traces of dissolved O_2 . Illumination was provided by a 150 w tungsten reflector flood lamp. The light was filtered through one thickness of Cinemoid (Strand Electric Ltd., London, W.C. 2) deep-orange sheeting, and through 2 in. of water. Chloroplast particles (P_1S_2) were prepared by the method of Whatley & Arnon (1963), except that grinding

with sand was replaced by 15 sec. blending in an MSE Ato-Mix blender. Their ability to evolve oxygen was destroyed either by heating for 5 min. at 55° or by a brief exposure to ultrasonic oscillation, with the Dawe Soniprobe at setting 4 for 10 sec. NADP was purchased from Sigma (London) Chemical Co. (London, S.W. 6) or Boehringer Corp. (London) Ltd., London, W. 5. Benzyl viologen was from British Drug Houses Ltd. (Poole, Dorset). Tris and glucose oxidase were from Sigma (London) Chemical Co. DEAE-cellulose was Whatman DE 23. Other reagents were A.R. grade.

RESULTS

Ferredoxin was reduced by illuminated chloroplasts with reduced dichlorophenol-indophenol as electron donor. The dye was kept in the reduced state by an excess of ascorbate in experiments with NADP as acceptor, or of cysteine in experiments with benzyl viologen as acceptor. The course of the reduction was followed by measuring changes in E_{420} for plant ferredoxins, or in E_{415} for bacterial ferredoxins. Prolonged illumination was found to cause destruction of the ferredoxin. Illumination periods were therefore kept as short as possible, less than 5 min. for plant ferredoxins and up to 15 min. for *Chromatium* ferredoxin. The reduced ferredoxin was stable under the conditions used.

Table 1 shows the results of an experiment in which spinach ferredoxin was reduced and then reoxidized in the dark with NADP. This experiment, in agreement with the results of Whatley *et al.* (1963), showed that two moles of spinach ferredoxin were reoxidized for each mole of NADP reduced, and that spinach ferredoxin is a one-electron acceptor. Table 2 shows a similar experiment in which benzyl viologen was used as electron acceptor. Benzyl viologen was found to show a large change in extinction in the 415–420 m μ region

Table 1. *Stoichiometry of the oxidation of spinach ferredoxin by NADP*

The reaction mixture contained tris-HCl buffer, pH 7.3 (0.2 M-mole), sodium ascorbate (20 μ moles), dichlorophenol-indophenol (0.2 μ mole), glucose (50 μ moles), glucose oxidase (1.0 mg.), spinach ferredoxin (2.0 mg.), chloroplasts equivalent to 0.05 mg. of chlorophyll and NADP (2.0 μ moles), in a total volume of 3.0 ml. of water. The experimental procedure is described in the text.

	E_{420}	E_{340}
Oxidized ferredoxin	0.62	0.77
Photoreduced ferredoxin	0.32	0.64
Ferredoxin reoxidized by NADP	0.61	0.93
ΔE due to ferredoxin	0.30	
ΔE due to NADPH ₂		0.16
Ferredoxin reoxidized (μ mole) ...		0.18
NADP reduced (μ mole) ...		0.08
Ratio ferredoxin:NADP ...		2.2

Table 2. *Stoichiometry of the oxidation of spinach ferredoxin by benzyl viologen*

The experimental conditions were as in Table 1, except that the ascorbate was replaced by cysteine (20 μ moles) and the NADP by benzyl viologen (2.0 μ moles).

	E_{420}	E_{600}
Oxidized ferredoxin	0.37	0.08
Photoreduced ferredoxin	0.14	0.04
Ferredoxin reoxidized by benzyl viologen	0.55	0.49
Air admitted	0.39	0.11
ΔE due to ferredoxin	0.25	
ΔE due to benzyl viologen		0.38
Ferredoxin oxidized (μ mole) ...		0.15
Benzyl viologen reduced (μ mole) ...		0.17
Ratio ferredoxin:benzyl viologen...		0.9

on reduction; the change is approximately one-third of the change at 600 $m\mu$ at which the reduction of benzyl viologen was measured. In the present experiments it was assumed that the ferredoxin was reoxidized only by the benzyl viologen. After measurement of the amount of benzyl viologen reduced, air was admitted to reoxidize the benzyl viologen, and the extinction at 420 $m\mu$ was read to check that the ferredoxin had not been destroyed by light. This experiment showed that one mole of ferredoxin reduced one mole of benzyl viologen. As benzyl viologen accepts one electron on reduction (Clark, 1960) this experiment also indicates that spinach ferredoxin is a one-electron carrier.

Similar experiments were also carried out with cytochrome *c* as electron acceptor. These also indicated that spinach ferredoxin carried one electron. However, with cytochrome *c* it is not possible to measure the amount of ferredoxin reoxidized. These experiments are not therefore reported in detail. Cytochrome *c* could not be used with bacterial ferredoxins as it is rapidly reduced by the oxidized forms of these ferredoxins.

Experiments with *A. nidulans* ferredoxin were done only with NADP as acceptor. It was found to be a one-electron carrier.

Tables 3 and 4 show similar experiments with *Cl. welchii* ferredoxin with NADP and benzyl viologen as final electron acceptor. The chloroplasts used in these experiments and in those with *Chromatium* ferredoxin were first treated by ultrasonic oscillation. It was found that when bacterial ferredoxin was reoxidized by NADP in the presence of untreated chloroplasts, a reaction dependent on the ferredoxin-NADP reductase of the chloroplasts, a change in extinction occurred due to changes in light-scattering. The reason for this change, which is most marked with *Chromatium* ferredoxin, has not been identified. It was, how-

Table 3. *Stoichiometry of the oxidation of Cl. welchii ferredoxin by NADP*

The experimental conditions were as in Table 1, except that the spinach ferredoxin was replaced by *Cl. welchii* ferredoxin (0.4 mg.).

	E_{415}	E_{340}
Oxidized ferredoxin	0.64	0.86
Photoreduced ferredoxin	0.32	0.80
Ferredoxin reoxidized by NADP	0.56	0.95
ΔE due to ferredoxin	0.24	
ΔE due to NADPH ₂		0.09
ΔE due to NADPH ₂ corrected for partial reoxidation of ferredoxin		0.105
Ferredoxin oxidized (μ mole) ...		0.053
NADP reduced (μ mole) ...		0.052
Ratio ferredoxin:NADP ...		1.0

Table 4. *Stoichiometry of the oxidation of Cl. welchii ferredoxin by benzyl viologen*

The experimental conditions were as in Table 2, except that the spinach ferredoxin was replaced by *Cl. welchii* ferredoxin (0.35 mg.).

	E_{415}	E_{600}
Oxidized ferredoxin	0.51	0.10
Photoreduced ferredoxin	0.30	0.09
Ferredoxin reoxidized by benzyl viologen	0.59	0.32
Air admitted	0.52	0.11
ΔE due to ferredoxin	0.21	
ΔE due to benzyl viologen		0.22
Ferredoxin oxidized (μ mole) ...		0.047
Benzyl viologen reduced (μ mole) ...		0.093
Ratio ferredoxin:benzyl viologen...		0.5

ever, prevented by ultrasonic treatment of the chloroplasts. The experiment with NADP (Table 3) showed that one mole of NADP was reduced for one mole of ferredoxin oxidized, and the experiment with benzyl viologen (Table 4) that two moles were reduced for each mole of ferredoxin oxidized. *Cl. welchii* ferredoxin was not completely reoxidized by NADP, the reoxidation varying between 80 and 90%. Under identical conditions 100% reoxidation by oxygen was obtained. As there is a large extinction change at 340 $m\mu$ on reduction of this ferredoxin, a correction for the percentage recovery of extinction at 340 $m\mu$ based on that observed at 415 $m\mu$ was made. Tagawa & Arnon (1968) used a control cuvette in which the ferredoxin was reoxidized by air to correct for this change. Since *Cl. welchii* ferredoxin is not completely reoxidized by NADP, this would not be a valid control in the present experiments. Tables 5 and 6 show experiments with *Chromatium* ferredoxin with NADP

and benzyl viologen as electron acceptor. As with *Cl. welchii* ferredoxin, stoichiometry of one mole of NADP reduced/mole of *Chromatium* ferredoxin oxidized or two moles of benzyl viologen reduced/mole of *Chromatium* ferredoxin oxidized was observed. These results indicate that *Chromatium* ferredoxin is also a two-electron carrier. Table 7

contains a summary of the results obtained in a number of experiments with each ferredoxin.

DISCUSSION

Ferredoxins are a group of non-haem iron proteins involved in electron-transfer reactions requiring a lower-potential electron carrier than nicotinamide nucleotides. Ferredoxins in bacteria are involved in hydrogen and nitrogen metabolism, pyruvate metabolism and photosynthesis (Malkin & Rabinowitz, 1967). Plant ferredoxins are involved in photoreduction and phosphorylation reactions (Arnon, 1965). Plant and bacterial ferredoxins are normally interchangeable as carriers in the different systems, though plant ferredoxins are less effective than bacterial ferredoxins in bacterial systems and vice versa (Evans & Buchanan, 1965; Bachofen & Arnon, 1966). All ferredoxins are chemically similar in that they contain iron and inorganic sulphur in stoichiometric amounts. They differ in that plant ferredoxins contain two atoms of iron and two atoms of sulphur/molecule whereas bacterial ferredoxins contain four or more atoms of iron and of sulphur/molecule. *Chromatium* ferredoxin was reported to contain only three atoms of iron and sulphur/molecule (Bachofen & Arnon, 1966); this value was, however, based on a molecular weight of 6000, whereas the correct molecular weight appears to be 9000–10000 (Sasaki & Matsubara, 1967). Bacterial and plant ferredoxins show similar electron-spin-resonance spectra when reduced chemically or photosynthetically (D. O. Hall, M. C. W. Evans, J. F. Gibson & F. R. Whatley, unpublished work), indicating that the mechanism of reduction is similar.

Our results, in agreement with those of Whatley *et al.* (1963) and Horio & San Pietro (1964), show that spinach ferredoxin is a one-electron carrier, as is the spectroscopically similar *A. nidulans* ferredoxin. Our results with *Cl. welchii* and *Chromatium* ferredoxins agree with those of Sobel & Lovenberg (1966) in showing that bacterial ferredoxins are two-electron carriers. Tagawa & Arnon

Table 5. *Stoichiometry of the oxidation of Chromatium ferredoxin by NADP*

The experimental conditions were as in Table 1, except that the spinach ferredoxin was replaced by *Chromatium* ferredoxin (0.5 mg.).

	E_{415}	E_{340}
Oxidized ferredoxin	0.50	0.73
Photoreduced ferredoxin	0.31	0.70
Ferredoxin reoxidized by NADP	0.50	0.82
ΔE due to ferredoxin	0.19	
ΔE due to NADP		0.09
Ferredoxin oxidized (μ mole) ...		0.046
NADP reduced (μ mole) ...		0.045
Ratio ferredoxin:NADP ...		0.99

Table 6. *Stoichiometry of the oxidation of Chromatium ferredoxin by benzyl viologen*

The experimental conditions were as in Table 2, except that the spinach ferredoxin was replaced by *Chromatium* ferredoxin (0.8 mg.).

	E_{415}	E_{600}
Oxidized ferredoxin	1.14	0.29
Photoreduced ferredoxin	0.77	0.25
Ferredoxin reoxidized by benzyl viologen	1.17	0.54
Air admitted	1.04	0.29
ΔE due to ferredoxin	0.27	
ΔE due to benzyl viologen		0.25
Ferredoxin reoxidized (μ mole) ...		0.057
Benzyl viologen reduced (μ mole) ...		0.12
Ratio ferredoxin:benzyl viologen ...		0.47

Table 7. *Number of electrons transferred to plant and bacterial ferredoxins*

Source of ferredoxin	Electron acceptor	No. of determinations	No. of electrons (range)
Spinach (green plant)	NADP	3	0.7–1.2
Spinach	Benzyl viologen	5	1.0–1.3
<i>A. nidulans</i> (blue-green alga)	NADP	3	0.75–1.0
<i>Cl. welchii</i> (anaerobic bacterium)	NADP	6	1.75–2.4
<i>Cl. welchii</i>	Benzyl viologen	4	1.85–2.2
<i>Chromatium</i> (photosynthetic bacterium)	NADP	6	1.7–2.2
<i>Chromatium</i>	Benzyl viologen	3	1.72–2.1

(1968) obtained results suggesting that bacterial ferredoxins are one-electron carriers. We have used both the methods that they described for measuring the stoichiometry of electron transfer by ferredoxin in experiments with spinach and bacterial ferredoxins. We find that with both the methods described we obtain results showing that spinach ferredoxin is a one-electron carrier and bacterial ferredoxins are two-electron carriers. The most serious difficulties experienced in making these measurements are the destruction of the ferredoxin during the experiment and the failure to remove traces of oxygen from the gas phase. The first difficulty can be overcome by basing calculations on the amount of ferredoxin reoxidized rather than the amount reduced, and by checking that on reoxidation all the ferredoxin is recovered. Failure to remove traces of oxygen from the gas phase will result in reoxidation of all or part of the ferredoxin by oxygen rather than by the added electron acceptor. This will result in low values for the number of electrons carried by ferredoxin. It therefore seems likely that any error in these experiments will tend to give a low rather than a high value for the number of electrons transferred.

In view of the comparative results that we have obtained with plant and bacterial ferredoxins and the agreement of these results with those of other workers, we conclude that plant and bacterial ferredoxins differ in the number of electrons that they carry, plant ferredoxins accepting one and bacterial ferredoxins two electrons.

Electron-spin-resonance data suggest that the mechanisms of reduction of bacterial and plant ferredoxins are similar. Our results are therefore consistent with the proposal of Gibson, Hall, Thornley & Whatley (1966) that the active centre of ferredoxin involves the acceptance of one electron by a complex containing two iron and two sulphur

atoms, and that plant ferredoxins contain one such complex and bacterial ferredoxins contain two complexes.

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