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A Four-Iron Ferredoxin from *Desulfovibrio desulfuricans*

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Ferredoxin from *Desulfovibrio desulfuricans* was isolated, purified and crystallized. It contains four iron atoms and four sulphido or 'acid-labile' sulphur atoms for a molecule of 6000 daltons. The absorption spectrum in the u.v.–visible region and the electron-paramagnetic-resonance signals of the reduced protein are similar to those observed for other four-iron ferredoxins. The amino acid composition is different from that of *Desulfovibrio gigas* ferredoxin. The redox potential of −0.33 V at pH 7.0 was determined by dye techniques.

Although the class of eight-iron–eight-sulphido ferredoxins has been extensively studied (see Mason & Zubeita, 1973, and references cited therein) and structurally characterized in the case of *Peptococcus aerogenes* ferredoxin (Sieker *et al.*, 1972), only three examples of four-iron bacterial ferredoxin are known: those in *Desulfovibrio gigas* (LeGall & Dragoni, 1966), *Bacillus polymyxa* (Shethna *et al.*, 1971) and *Spirochaeta aurantia* (Johnson & Canale-Parola, 1973). We now report the isolation, purification and physicochemical properties of a four-iron ferredoxin from *Desulfovibrio desulfuricans*.

Methods

Growth of bacteria. Desulfovibrio desulfuricans strain Berre S (N.C.I.B. 8388) was grown in a modification of medium C (Postgate, 1966) in 400-litre all-glass fermentors and harvested by continuous-flow centrifugation.

Extraction and purification of ferredoxin. A 200g wet wt. batch of bacteria was suspended in 500ml of 50mM-Tris–HCl buffer, pH 7.6, and disrupted in a French pressure cell at 31 MPa (2 tons/in^2). The resultant viscous extract was centrifuged at 23000*g* for 30min at 4°C, and all subsequent manipulations were carried out at 4°C. The greenish supernatant was treated batchwise with 150ml of Whatman DE-52 DEAE-cellulose, equilibrated in 10mM-Tris–HCl buffer, pH 7.6. The DEAE-cellulose was packed as a column, washed with 500ml of 50mM-Tris–HCl buffer, pH 7.6, and the adsorbed proteins were eluted with 50mM-Tris–HCl buffer, pH 7.6, containing 0.5M-NaCl, to yield approx. 100ml of green–brown solution, which was concentrated to 60ml by passing a stream of N_2 over the solution for 20h. This material was then passed through a Sephadex G-50 column (2.5cm × 50cm) at 50ml/h, resulting in a separation of the green desulfoviridin from the red–brown band that was eluted slowly and contained ferredoxin and the rubredoxin described by Newman & Postgate (1968).

The resulting 150ml of solution was applied to a Whatman DE-52 DEAE-cellulose column (2cm × 35cm), equilibrated with 50mM-Tris–HCl buffer, pH 7.6. The NaCl concentration was increased successively to 0.05, 0.10, 0.15, 0.20 and 0.25M. Rubredoxin separated and was eluted as a sharp red band at 0.2M-NaCl. Although the brown ferredoxin began to be eluted slowly at 0.2M-NaCl, it was eluted at this stage with 50mM-Tris–HCl buffer, pH 7.6, containing 0.25M-NaCl at 100ml/h.

The ferredoxin preparation had a strong absorption at 260nm (E_{400}/E_{260} ratio 0.05). Attempts to remove this impurity by pH gradient and preparative-scale disc gel electrophoresis were unsuccessful. Slow elution from DEAE-cellulose columns (2cm × 50cm) with 50mM-Tris–HCl buffer, pH 7.6, containing 0.2M-NaCl and subsequent removal of the DEAE-cellulose through which the ferredoxin had been eluted with a pipette (Mitsui, 1971) yielded preparations with an E_{400}/E_{260} ratio of 0.25. Complete removal of the 260nm-absorbing material could be accomplished only by passing the material through a calcinated alumina column (LeGall & Dragoni, 1966) equilibrated with 50mM-Tris–HCl buffer, pH 7.6. After this treatment the ferredoxin was eluted once again from DEAE-cellulose and desalted on a Sephadex G-50 column. Addition of $(NH_4)_2SO_4$ crystals to this solution yielded fine needle-like crystals approx. 0.1mm long; these were too fine for X-ray-crystallographic examination.

Iron and acid-labile sulphide analysis. Iron was determined colorimetrically (Sven & Peterson, 1958) and acid-labile sulphide by the method of Gilboa-Garber (1971).

Molecular-weight determination. (*a*) Thin-layer plates of Sephadex G-50 (superfine grade) were prepared and run at room temperature with standards as described by Dalton & Zubieta (1973).

(*b*) Molecular weight was also determined by the approach-to-sedimentation equilibrium technique (Schachman, 1957) by employing a Beckman–Spinco model E ultracentrifuge equipped with schlieren optics and at a temperature of 20±0.2°C. The partial specific volume was assumed to be 0.63ml/g, a value representative of bacterial ferredoxins (Lovenberg *et al.*, 1963).

Amino acid analyses. Acid hydrolyses on 0.3mg of pure ferredoxin were performed for 24 and 72h as previously described (Dalton & Zubieta, 1973). Performic acid oxidations were performed by the method of Moore (1963).

Molar extinction coefficient. The molar extinction coefficient was determined by dry-weight analysis of pure samples of ferredoxin (Hong & Rabinowitz, 1970).

Disc gel electrophoresis. The method of Hendrick & Smith (1968) was used to prepare gels of 15% polyacrylamide concentration at pH8.5 and 4.5.

Measurement of redox potential and the number of electrons carried. The number of electrons required to reduce 1g-atom of ferredoxin was calculated by integration of e.p.r. (electron-paramagnetic-resonance) signals that result on reduction with $Na_2S_2O_4$, obtained with a Varian E9 spectrometer with temperature control as described by Lowe *et al.* (1972).

The oxidation–reduction potential was measured by using the Safranine dye method as described by Lovenberg & Sobel (1965).

Results and discussion

The ferredoxin samples used for characterization were judged to be pure on the bases of single bands on polyacrylamide gels as described above, the absence of 260nm-absorbing contaminant, and E_{400}/E_{280} ratio of 0.78 after recrystallization and the absence of $g = 4.3$ signals from the e.p.r. spectrum of the oxidized samples.

Table 1. *Amino acid compositions of ferredoxins from D. desulfuricans, D. gigas, S. aurantia and P. aerogenes*

	D. desulfuricans				
	Nearest integer from hydrolysis*	Nearest integer from oxidation†	*D. gigas*‡	*S. aurantia*§	*P. aerogenes*‖
Asp	10	10	11	8	8
Thr¶	3	3	0	2	0
Ser¶	2	2	3	3	5
Glu	10	11	9	6	4
Pro	3	3	4	5	5
Gly	6	6	1	3	4
Ala	2	2	6	8	7
Val**	5	5	5	4	4
Met	0	0	2	0	0
Ile**	4	4	5	4	6
Leu	2	2	1	1	0
Tyr	0	0	0	2	2
Phe	0	0	1	1	0
His	1	1	0	0	0
Lys	2	2	1	3	1
Arg	0	0	1	0	0
Cys	6	6‡‡	6	6	8
Trp††	0	0	0	0	0
Total	56	57	56	56	54
Fe	4	4	4	4	8
S^{2-}	4	4	4	4	8

* The results are the average of values obtained for 24 and 72h of hydrolysis at 110°C.
† The results for the performic acid oxidation are those for 48h at 110°C.
‡ Travis *et al.* (1971).
§ Johnson & Canale-Parola (1973).
‖ See Tanaka *et al.* (1971) for the results on this and a variety of eight-iron–eight-sulphido ferredoxins.
¶ In determining the values for threonine and serine, the results were extrapolated to zero time.
** The best ratio for valine and isoleucine is that based on the 72h recovery.
†† Determined by the method of Opienska-Blauth *et al.* (1963).
‡‡ Determined as cysteic acid.

The u.v.–visible spectrum is similar in general appearance to that reported for other four-iron and eight-iron bacterial ferredoxins (Tsibris & Woody, 1970), showing absorption bands at 280 and 400nm (as opposed to 390nm in most cases) and shoulders at 290 and 320nm. The molar extinction coefficient, ϵ_{400}, was 31000. Addition of excess of $Na_2S_2O_4$ to a solution of the sample resulted in the loss of the 400nm absorption within 2–3min.

A molecular weight of 6100 was calculated on the basis of sedimentation-equilibrium experiments. A plot of distance migrated on a thin-layer Sephadex gel against the logarithm of the molecular weight gave a value of approx. 6200. The molecular weight based on the amino acid composition and the iron and sulphide content was 6300 (see below). These values are consistent and give an average molecular weight for *D. desulfuricans* ferredoxin of 6200 ± 100.

There are 4g-atoms of iron and 4g-atoms of sulphide per mol of *D. desulfuricans* ferredoxin. These analytical values for the molecular weight and the iron and sulphide contents are comparable to those obtained from the ferredoxin from *D. gigas* (Travis *et al.*, 1971).

The amino acid composition is presented in Table 1 and compared with that obtained for the typical four-iron–four-sulphido ferredoxins from *D. gigas* and *Spirochaeta aurantia* and the eight-iron–eight-sulphido ferredoxin from *P. aerogenes*. The analysis is significantly different from that of *D. gigas* ferredoxin in that no methionine residues are present; it is also surprising that neither phenylalanine nor tyrosine is present. The significance of this result on the theories advanced for electron transfer via intermediacy of aromatic residues (Mason & Zubieta, 1973) is not yet clear, since it is apparent from the studies on the sequence of *Clostridium tartarivorum* ferredoxin (Tanaka *et al.*, 1971) that histidine will replace tyrosine or phenylalanine in the sequence of eight-iron–eight-sulphido ferredoxins. As with both previously reported four-iron–four-sulphido ferredoxins, there are six cysteine residues, a result indicating that further structural investigations on this class of ferredoxins would be rewarding.

The e.p.r. experiments resulted in a rhombic signal on reduction with dithionite. Integration of the signal revealed that approx. 1 electron equivalent was accepted per 6000-dalton unit. Fig. 1 compares the e.p.r. signals for fully reduced *D. desulfuricans* ferredoxin with the signals obtained for totally reduced (2 electron equivalents) and partially reduced (1 electron equivalent) ferredoxin from *Veillonella alcalescens* (Dalton & Zubieta, 1973).

The e.p.r. signal of fully reduced *D. desulfuricans* ferredoxin resembles that of *Bacillus polymyxa* (Shethna *et al.*, 1971). It was also similar to that of the half-reduced ferredoxin from *V. alcalescens*, consistent with it containing a single Fe_4S_4 cluster capable of accepting 1 electron equivalent/mol. This cluster geometry for the iron–sulphido groups has been observed in both *Chromatium* high-potential iron protein and *P. aerogenes* ferredoxin (Carter *et al.*, 1972). The E_0' value for *D. desulfuricans* ferredoxin was in the range of Safranine, the value at pH7.0 being −330mV for a one-electron process.

The ferredoxin from *D. desulfuricans* thus falls into

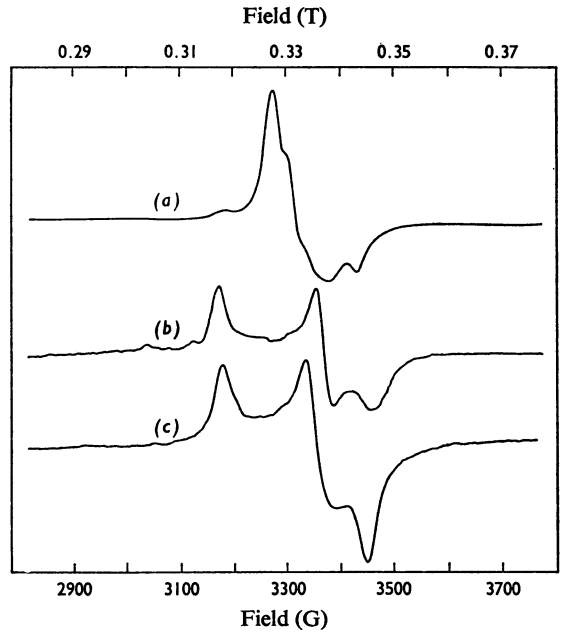

Fig. 1. *E.p.r. spectra for (a) fully reduced V. alcalescens ferredoxin, (b) V. alcalescens ferredoxin with the addition of* 0.6 *electron equivalents and (c) fully reduced D. desulfuricans ferredoxin*

Samples (0.2ml) containing 0.52μmol of ferredoxin/ml of 50mM-potassium phosphate buffer or 10mM-Tris–HCl buffer, pH7.8, were made anaerobic in evacuable e.p.r. tubes by repeated gassing with argon +hydrogen (98:2) followed by evacuation. To each tube was added an anaerobic solution of 42.8mM-$Na_2S_2O_4$ to give up to two electrons/molecule of *V. alcalescens* ferredoxin. The $Na_2S_2O_4$ solution was standardized before and after use against standard $K_3Fe(CN)_6$ with Methyl Viologen indicator. The samples were frozen in liquid N_2 after mixing for 1min. The e.p.r. conditions were as follows: frequency, 100kHz; microwave power, 20mW; scan time, 2min; time-constant, 0.1s; temperature, 21°K; modulation amplitude, 0.001T (10G); operating frequency, 9.143GHz; receiver gain, 4×10^1 for (*a*), 10^2 for (*b*) and (*c*); field in teslas and gauss as indicated.

the class of four-iron–four-sulphido ferredoxins (Mason & Zubieta, 1973). Only the four-iron ferredoxin of *D. gigas* has so far had its amino acid sequence determined; its polypeptide chain shares characteristics with both plant and bacterial ferredoxins, and this may indicate a primitive character (Hall *et al.*, 1971). The presence of a single Fe_4S_4 cluster may also be a primitive character among bacteria in view of the fact that the genus *Desulfovibrio* is believed to be 'ancient' (Klein & Cronquist, 1967).

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