THE PLANT FERREDOXINS AND THEIR RELATIONSHIP TO THE EVOLUTION OF FERREDOXINS FROM PRIMITIVE LIFE

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ABSTRACT

The first few ferredoxins were isolated, more or less simultaneously, from *Clostridum* and spinach. These non-haem, iron-containing oxidoreductases had almost the same low redox potential and both could catalyse the photoreduction of NADP by chloroplasts, but differed in molecular weight and in the number of iron atoms they contained. Since that time nearly forty more ferredoxins have been isolated from all classes of organisms. These fall into four categories containing respectively 2, 4, 6 or 8 atoms of iron per mole. Numerous physical properties have been determined and studies on circular dichroism, electron spin resonance, proton magnetic resonance and Mössbauer spectra have been especially valuable in giving an insight into the varying nature of the active centre of the different ferredoxins. The amino acid sequences of fourteen ferredoxins have been determined and together with the variation in function permit phylogenetic relationships to be determined. The experimental work leading to these results is covered in this review.

INTRODUCTION

The name ferredoxin was originally suggested by Mortenson *et al*[1] for an iron-containing protein which they isolated from the bacterium *Clostridium pasteurianum* and which functioned as an electron carrier in the phosphoroclastic reaction leading to hydrogen formation in this organism. About the same time Tagawa and Arnon[2] isolated a non-haem iron protein with a low oxidation–reduction potential from spinach leaves which catalyzed the reduction of NADP to $NADPH_2$ by illuminated chloroplasts. These authors also found that *Cl. pasteurianum* ferredoxin could substitute for the spinach protein in the photoreduction of NADP. Though the spinach non-haem iron protein differed from baterial ferredoxin in iron content, in molecular weight and in visible absorption spectrum, the similarity of the two proteins in their low redox potentials (close to that of the hydrogen electrode) and in their interchangeability in the photo-reduction of NADP by chloroplasts, caused Tagawa and Arnon to propose that the term ferredoxin be extended to include all non-haeme iron proteins with an oxidation–reduction potential more negative than NADP. Proteins similar to spinach ferredoxin had previously been reported in the literature under various names such as the methaemoglobin reducing factor, photosynthetic pyridine nucleotide reductase (PPNR), red enzyme of *Chlorella* and so on.[3]

Table 1. Properties of ferredoxins

	Fe or S atoms mol^{-1}	Molecular weight	Number of amino acids	Redox potential E'_0, mV	Number of electrons transferred	EPR signal (red. or ox.) temp, K	Refs.
8 Fe ferredoxins							
Clostridium (anaerobic fermentor)	8	6000	55	−395	2	red. 15	76, 77, 2, 16, 78
Chlorobium (green photosynthesizer)	8	6000	56	—	–	red. 15	79, 73
Chromatium (red photosynthesizer)	8	10 000	81	−490	2	red. 15	79, 62, 80, †
6 Fe ferredoxins							
Azotobacter Type III (aerobic N_2 fixer)	6	20 000	171	—	–	—	81, 83
	6–7	13 000	—	—	–	ox. 77	
Rhodospirillum Type I (red facultative photosynthesizer)	6	8700	76	—	–	—	79
4 Fe ferredoxins							
Desulphovibrio (sulphate reducer)	4	6000	56	—	1	red. <30?	66
Bacillus polymyxa (facultative N_2 fixer)	4	9000	—	−380	1	red. <30	67, 68
Beef heart mitochondria (subunit of succinate dehydrogenase)	4	70 000	—	—	–	red.* 103	84
Chromatium HiPIP (red photosynthesizer)	4	9650	86	+350	1	ox. 77	64, 65, 85
2 Fe ferredoxins							
Bacterial							
Clostridium EPR protein (anaerobic N_2 fixer)	2	24 000	—	—	1	red. 77	86, 46
Clostridium ozoferredoxin	2	27 500	—	—	–	red. 15?	87

Clostridium hydrogenase	2	60 000	—	—	–	—	88, 89
E. coli	2	12 600	—	—	–	red. 77	91,†
Rhodospirillum Type II (red photosynthesizer: facultative aerobe)	2	7500	67	—	–	—	79
Azotobacter Type I (aerobic N_2 fixer)	2	21 000	182	—	1	red. 104	82
Azotobacter Type II (aerobic N_2 fixer)	2	24 000	—	—	1	red. 77	82, 90
Pseudomonas putida (aerobic hydroxylator)	2	12 500	114	−240	1	red. 77	35, 54
Plant							
Microcystis (blue-green alga)	2	11 500	98	—	1	red. 77	92
Nostoc (blue-green alga, N_2 fixer)	2	12 000	—	−406	–	—	93
Scenedesmus (green alga)	2	11 500	96	—	1	red. 77	14, †
Equisetum (horsetail, primitive plant)	2	11 500	95	—	–	red. 77	50, †
Spinacea (higher plant)	2	11 500	97	−430	1	red. 77	2, 94, 16, 29, 30
Animal							
Beef adrenal glands (adrenodoxin)	2	13 000	115	−340	1	red. 77	95, 55, 45
Beef heart mitochondria (Complex III Fe-S protein)	2	26 000	—	+220	1	red. 97	96

* EPR signal seen in purified succinate dehydrogenase.
† Our unpublished data.

In the early stages of the study of the ferredoxin two distinct types were recognised, the bacterial ferredoxins and the chloroplast ferredoxins. However, as we shall see later, with the availability of information regarding the chemical composition, amino acid sequence and physicochemical parameters of ferredoxins, and with the discovery of more than one ferredoxin in some organisms, it became necessary to revise this simple classification. Furthermore, proteins resembling ferredoxins in structure and composition were also obtained from mammalian sources. It is now recognised that the ferredoxins belong to the group known as iron–sulphur proteins (proteins which contain one or more atoms of non-haem iron bonded to cysteine or inorganic sulphur atoms) which are ubiquitous in nature. Since they occur in primitive bacteria, algae, higher plants and animals, ferredoxins are very good candidates for the study of biological evolution based on variations in amino acid sequences of homologous proteins. This technique has been applied in the phylogenetic classification of organisms from the primary structures of several proteins including cytochromes *c*, haemoglobins and fibrinopeptides[4–8].

The main features of the ferredoxins are the presence of an active centre containing iron and sulphur, their low redox potential ($E'_0 = c - 0.4$ volts), their participation in biological electron transport, and the formation of a characteristic epr signal centred around $g = 1.94$ (in the reduced state) at very low temperatures, e.g. liquid nitrogen or liquid helium. The types of electron transfer reactions in which they participate are quite diverse, such as the *Clostridia* phosphoroclastic reaction, nitrogen fixation, nitrite reduction, photosynthesis and mammalian steroid hydroxylation[9–11]. Some properties of the various ferredoxins are listed in *Table 1*.

EXTRACTION OF FERREDOXINS

The ferredoxins characteristically contain a preponderance of acidic amino acids and therefore are readily adsorbed by the anion exchange resin DEAE-cellulose. This property is well utilized in the isolation and purification of these proteins. The main steps involved in the isolation procedure are: (1) preparation of a cell-free extract and centrifugation to get rid of insoluble debris; (2) adsorption of ferredoxins from the supernatant by DEAE-cellulose either on a column or by batchwise addition of the resin; (3) chromatographic elution of the ferredoxin from the DEAE-cellulose using a buffered sodium chloride gradient; (4) ammonium sulphate fractionation; and (5) gel filtration on Sephadex G-75 or Biogel P-10[12, 13]. Common contaminants in ferredoxins are nucleic acid derivatives which can be removed by protamine sulphate treatment, by treatment with nucleases or by chromatography on hydroxylapatite[14]. Many ferredoxins are sensitive towards heat and aerobic oxidation and so over long periods it is advisable to store these proteins in liquid nitrogen.

THE PLANT-TYPE FERREDOXINS

The plant-type ferredoxins are obtained from all oxygen-evolving photosynthetic organisms, from the blue-green algae up to higher vascular plants.

Table 2. Amino acid composition of plant ferredoxins

	Lys	His	Arg	Trp	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Total	Ref.
Microcystis (blue-green alga)	3	1	1	0	13	7	6	13	4	12	9	5	4	1	6	9	3	1	98	92
Anacystis (blue-green alga)	3	1	1	0	15	12	7	11	3	6	12	6	9	0	5	7	5	2	105	97
Bumelleriopsis (yellow-green alga)	5	1	1	0	14	6	7	15	3	6	11	4	6	1	5	9	4	3	101	98
*Scenedesmus** (green alga)	4	1	1	0	12	10	8	10	4	7	10	6	5	1	3	7	4	3	96	14
Euglena (green alga)	5	1	1	0	14	9	8	9	4	7	8	6	6	0	4	7	1	3	93	99
Cladophora (green alga)	7	1	2	0	10	8	7	16	3	7	12	2	7	2	3	9	4	1	101	100
Equisetum (primitive vascular plant)	4	1	1	0	9	7	8	16	4	9	6	4	6	1	5	8	2	4	95	50
Polystichum (fern)	4	2	1	0	14	6	8	9	5	9	7	5	5	2	6	7	3	4	97	101
Zea mays (monocot, C_4 plant)	3	2	1	1	13	5	8	14	4	8	8	4	10	0	5	8	5	1	100	102
Cyperus (nutsedge, C_4 plant)	6	1	1	1	10	4	7	14	4	9	8	4	9	1	4	10	4	1	98	103
*Colocasia** (taro, monocot)	5	1	1	1	10	6	8	15	4	9	6	5	10	0	4	6	4	2	97	104
Amaranthus (pigweed, C_4 plant)	4	1	1	1	12	8	8	14	5	6	10	5	5	1	6	6	4	1	98	101
*Spinacea** (spinach)	4	1	1	1	13	8	7	13	4	6	9	5	7	0	4	8	4	2	97	94
*Medicago** (alfalfa, lucerne)	5	2	1	1	9	6	8	16	3	7	9	5	9	0	4	6	4	2	97	20
Gossypium (cotton)	3	1	2	1	16	4	6	18	4	8	8	4	8	1	4	6	2	3	99	105
*Leucaena** (Koa; tree)	5	1	2	1	11	4	7	16	5	6	7	5	6	0	4	10	3	3	96	106

* Amino acid sequences known.

They contain two atoms of iron and two of labile sulphur per molecule, about 97 amino acid residues and have a molecular weight of about 12 000 daltons. They have a redox potential of about − 0.43 volts and they transfer one electron at a time during biological reactions[2, 15, 16]. The amino acid compositions of the plant ferredoxins available up to now are given in *Table 2*.

The major role of ferredoxins in the chloroplasts is to mediate the transfer of electrons from chlorophyll (or from the 'primary electron acceptor') to the flavoprotein enzyme, ferredoxin-NADP reductase, which in turn reduces NADP to $NADPH_2$. Thus the ferredoxin catalyzes reduction of NADP to $NADPH_2$ which forms part of the non-cyclic photophosphorylation pathway. Ferredoxin also acts as a catalyst in cyclic photophosphorylation[3] and in nitrite reduction in the chloroplasts[17]. The similarity of the ferredoxins in the physiological activity of all O_2-evolving photosynthetic organisms was first observed by Mitsui and Arnon[3] when they found that ferredoxin from the blue-green alga *Nostoc* functioned as effectively as spinach ferredoxin as a catalyst in the photoreduction of NADP by spinach chloroplasts. The activity of ferredoxins can be easily measured by adding catalytic amounts of the ferredoxin to an assay mixture containing spinach chloroplasts (washed to remove soluble ferredoxin) and NADP and measuring the rate of reduction of NADP to $NADPH_2$ on illumination (monitored as increase in absorbance of the reaction mixture at 340 nm)[18]. We have recently studied the chemical and spectroscopic properties of ferredoxins from the blue-green algae *Microcystis* and *Spirulina* and these are found to be similar to those of higher plant ferredoxins. These properties will be discussed in detail later.

PHYSICAL PROPERTIES OF FERREDOXINS

Optical spectra

The plant ferredoxins are red in colour and their optical spectra exhibit absorption maxima at about 465, 420, 330 and 278 nm (see *Figure 1*). Their molar absorbance at 420 nm is about 9 to 10 $\times$ 10^3 l cm^{-1}. The absorption of the protein in the visible region is mainly due to the iron–sulphur chromophore group and is lost on treatment of ferredoxins with reagents which remove iron or sulphur, i.e. mersalyl, dipyridyl, dilute acids, etc. The iron and sulphur can be put back and biologically active ferredoxin reconstituted by chemical methods[12, 19]. On reduction with sodium dithionite, 50 per cent of the visible absorption is lost but the original spectrum is restored on re-oxidation by shaking in air. The optical absorption at 278 nm is partly due to the aromatic amino acid content of the ferredoxins and partly to the presence of the chromophoric group. The ratio R of the absorption at 420 nm to that at 278 nm ($R = A_{420}/A_{278}$) is often characteristic for a particular ferredoxin and is taken as an index of the purity of the specimen. The R values of pure ferredoxins vary from about 0.47 (e.g. spinach ferredoxin containing one Trp and four Tyr residues) to 0.75 (e.g. *Equisetum* ferredoxin with two Tyr and no Trp residues). The R value decreases on exposure of ferredoxins to air and on long-term storage at room temperatures. In the case of alfalfa ferredoxin the decrease in R value on aerobic exposure is attributed to a loss in the 'labile sulphur content'[20] while with spinach ferredoxin it is

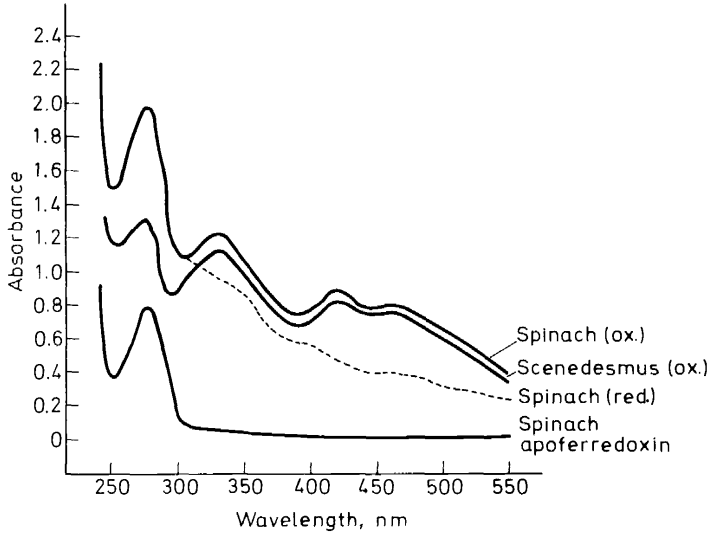


Figure 1. Absorption spectra of plant ferredoxins.

suggested that the labile sulphur atoms are oxidized to sulphur in the oxidation state zero covalently bound to protein[21].

Circular dichroism spectra

Circular dichroism (CD) spectra are probably more sensitive than the optical absorption spectra of proteins to changes which are brought about in the overall conformation of proteins[22]. The CD spectra of oxidized and reduced ferredoxins from three different species are shown in *Figure 2*. The overall shape of the spectrum is similar for the three ferredoxins. However, a closer examination reveals minor changes in the position and shape of the absorption bands for the different species. For example, the major peak at 428 nm is shifted slightly towards the red in the ferredoxins of the lower order plants (*Equisetum* and *Scenedesmus*) as is the shoulder at 460 nm. The shape of the major peak is slightly less wide in the *Zea mays* ferredoxin spectrum than that in the other two ferredoxins.

When we compared the spectra in a wide range of ferredoxins it was found that the spectra of the blue-green algal ferredoxins were all very similar, whereas those from higher plants showed greater variation in the detailed shape of the curves. The main absorption band around 428 nm in the CD spectra of ferredoxins is probably a reflection of the optical absorption at 420 nm. All these CD bands are attributed to charge transfer in the two Fe^{3+} atoms bonded to sulphur in the chromophoric group. Slight differences in the nature of the CD spectra in this region between various ferredoxins may be due to differences in the nature of the amino acid residues surrounding the chromophoric group. On reduction, the overall intensity of absorption decreases by about half, probably as a result of the

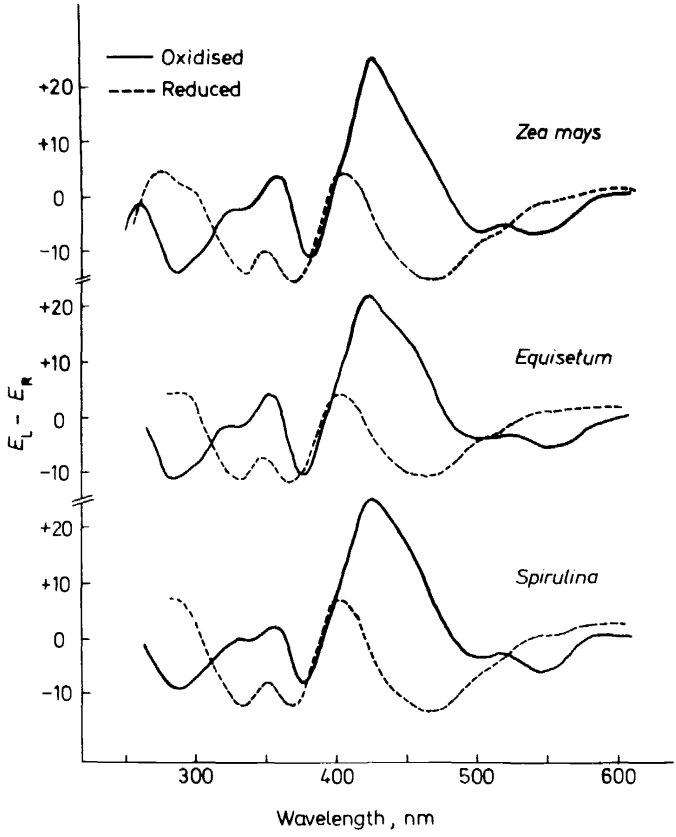


Figure 2. Circular dichroism of ferredoxins from three widely different plant species.

conversion of one Fe^{3+} to Fe^{2+}, which has a much smaller tendency to show charge transfer absorption in this region. The CD spectrum of apo-ferredoxin shows no absorption in the visible region.

Garbett *et al.*[22] were the first to detect an effect of 8M urea on the CD and ORD spectra of spinach ferredoxin. This was studied in further detail by other groups[23-25]. The process is complex. The first stage appears to be the conversion of ferredoxin to a form with a different protein configuration, and can be reversed by decreasing the concentrations of urea, or by adding 1M NaCl. Similar antagonism between the effects of urea and NaCl were observed in the epr spectrum by Cammack *et al*[25]. These changes in protein conformation may be similar to those which cause changes in the optical absorption and CD when ferredoxin forms a complex with ferredoxin NADP reductase[26, 27].

Eaton *et al.*[28] have investigated the nature of the iron sulphur complex in spinach ferredoxin and adrenodoxin by observing the CD and optical spectra in the near IR wavelength region. They identified d → d transitions of the iron atoms in the spectra of the reduced proteins at about 4000 cm^{-1}

and 6000 cm^{-1} and from the low energy of these transitions have concluded that the reduced iron–sulphur proteins contain a high spin ferrous ion in a distorted tetrahedral site.

Electron paramagnetic resonance spectra

One of the important physical properties of plant ferredoxins is that in the reduced state, and at low temperatures, they all exhibit an electron paramagnetic resonance (epr) spectrum centred around $g = 1.94$. The signal is characteristic of iron–sulphur proteins and the appearance of the signal (at temperatures ranging from liquid nitrogen to liquid helium) is used as a sensitive test to detect the presence of iron–sulphur proteins in whole cells and in cell-free preparations[9–11]. Palmer and Sands[29] and Hall *et al.*[30] showed that spinach ferredoxin when reduced with excess dithionite exhibited an epr signal centred at $g = 1.94$. Orme-Johnson and Beinert[31], by a series of anaerobic reductive titrations performed on a variety of iron–sulphur proteins, have shown that ferredoxins containing two iron atoms accept one electron on reduction; they also summarize other evidence for this stoichiometry.

The epr spectra of reduced plant ferredoxins exhibit orthorhombic symmetry. Typical spectra of two reduced ferredoxins are shown in *Figure 3* and the apparent g-values of a range of plant ferredoxins are given in *Table 3*.

Table 3. Principal apparent g-values of plant ferrodoxins at 77 K

Ferredoxin	g_x	g_y	g_z
Spinach	1.886	1.946	2.041
Parsley	1.893	1.948	2.039
Alfalfa	1.888	1.948	2.040
Maize	1.889	1.948	2.043
Equisetum	1.883	1.948	2.045
Scenedesmus	1.882	1.947	2.037
Bumilleriopsis	1.885	1.948	2.044
Microcystis	1.884	1.947	2.040
Anabaena	1.883	1.947	2.042
Spirulina	1.880	1.949	2.045

Pure specimens of oxidized ferredoxin exhibit no epr signal since it is non-magnetic. The similarity in the shape of the epr spectra from two such diverse plant species as parsley and *Spirulina* (*Figure 3*), and the nearness of the g-value parameters for so many ferredoxins is again indicative of the identity of the nature of the active centre of all these ferredoxins. The role of the iron and sulphur atoms at the active centre in the generation of the epr signal has been confirmed by a number of independent studies. A small broadening was observed in the epr spectrum of reduced ^{57}Fe substituted plant ferredoxins probably due to nuclear hyperfine interactions[12, 32, 33]. Fee and Palmer[34] replaced the labile sulphur of parsley ferredoxin with selenium isotopes and found that the shape of the epr spectrum of the selenium-substituted protein was different from that of the native protein

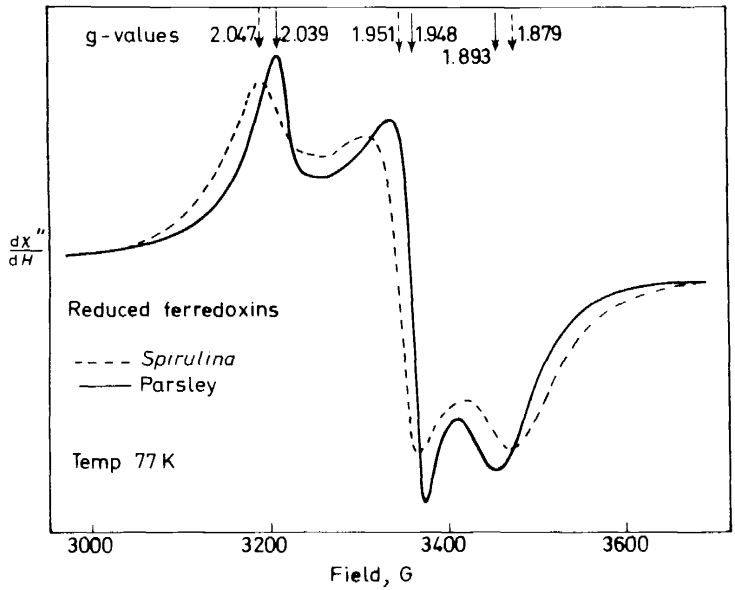


Figure 3. EPR spectra at 77 K of two reduced plant ferredoxins, illustrating the small variation in spectral shape which is observed. Other conditions of measurement were microwave frequency 9.17 GHz: modulation amplitude 4 G.

and tended toward axial symmetry, with a substantially narrower line width in the g_z region. This observation lends support to the view that the sulphur moiety of ferredoxin also contributes to the generation of the epr signals. Similar studies have been performed with adrenodoxin, putidaredoxin and the iron–sulphur proteins from *Azotobacter vinelandii* to establish the participation of iron and labile sulphur atoms in the production of the epr signal[35].

The shape and intensity of the epr signals of reduced plant ferredoxins are affected by the environment of the protein in solution. Thus Fee and Palmer[34] observed a broadening of the epr spectrum of parsley ferredoxin by chloride ions but not by sulphate or other salts. Parsley ferredoxin is the only ferredoxin in which this effect has been observed. The shape of the spectrum of reduced spinach ferredoxin in low concentrations was affected by the presence of urea up to a concentration of 5M, the g_z and g_y values moving slightly to higher magnetic fields and the g_x value moving downfield[24]. The ferredoxin is unstable in these conditions: unless care is taken to keep the ferredoxin–urea mixture perfectly anaerobic before reduction, the intensity of the epr signal decreases by as much as 50 per cent for a solution of spinach ferredoxin in 7.5M urea. Coffman and Stevens[36] found that the shape of the spectrum of reduced spinach ferredoxin in the presence of organic solvents like methanol tended towards axial symmetry. Similar changes in the epr spectral characteristics of ferredoxins were observed by treating ferredoxins with various concentrations of chaotropic agents, of

which urea is one example[25]. On adding low concentrations of the agents, e.g. 0.04M trichloroacetate, to reduced *Spirulina* ferredoxin there were decreases in the linewidth and small shifts in the apparent g-values until it resembled more closely that of parsley ferredoxin (*Figure 4*). The effect is reversed on adding 2M NaCl. It therefore seems that there is a balance between

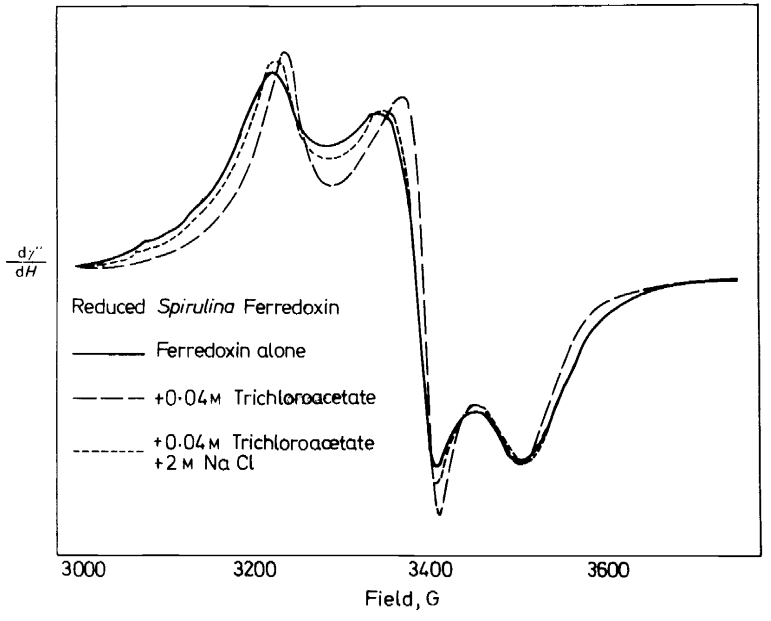


Figure 4. EPR spectra of *Spirulina* ferredoxin illustrating the effect of low concentrations of a chaotropic agent, and the reverse effect of NaCl. Conditions of measurement as in *Figure 3*.

the 'sharpening' effect of chaotropic agents, and the 'broadening' effect of chloride. Since ferredoxins are more stable in the presence of chloride it is tempting to speculate that those ferredoxins which have a broader epr spectrum in the reduced state are more stable than those with narrower line widths under equivalent conditions of salt concentration. Thus a comparison of the epr spectra of reduced parsely and *Spirulina* ferredoxins clearly shows that parsley ferredoxin has narrower linewidths (*Figure 3*) and therefore should be more sensitive to denaturing agents than the blue-green algal ferredoxin. Comparative stability studies[37] have shown that *Spirulina* ferredoxin is much more stable at room temperature than parsley ferredoxin (*Table 4*). Since the chromophoric groups in the two ferredoxins are identical, the reason for the difference in stabilities should be found in the differences in the amino acid compositions of these proteins.

The real cause of the induced changes in the epr spectral characteristics

Table 4. Relative activities of plant ferredoxins after storage for two months[37]

Species	% of original activity	
	Liquid N_2	Room temperature (21°C)
Spirulina	93	35
Scenedesmus	79	5
Spinach	81	3
Alfalfa	78	8
Parsley	74	3
Zea mays	81	15

of reduced ferredoxins by chloride ions, organic solvents and chaotropic agents is not yet definitely known. No corresponding changes have been observed in the CD or pmr spectra of the reduced ferredoxins in solution. One possible explanation is that the spectra were measured in frozen samples and the reagents might have induced small changes in the spectra by alteration of the structure of the ice around the ferredoxin molecule. On adding higher concentrations of chaotropic agents such as guanidine hydrochloride the epr spectrum undergoes a more dramatic change, which again is reversible[25]. This may be related to the changes in protein conformation which are seen in the CD.

As early as 1966 Gibson *et al.*[38] and Thornley *et al.*[39] proposed a model for the active centre of plant ferredoxins, based on epr studies and the magnetic susceptibility data then available. According to their postulate the two iron atoms in oxidized ferredoxin are high spin ferric antiferromagnetically coupled to give a net spin of zero and no epr signal; on reduction one

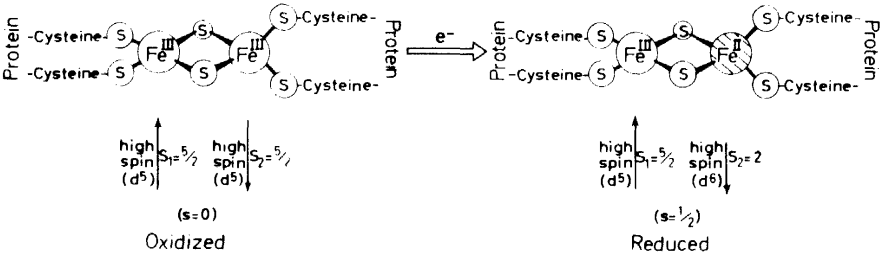


Figure 5. Model for the iron–sulphur group of oxidized and reduced plant ferredoxins[12].

of the iron atoms accepts an electron and becomes high spin ferrous to give a net spin of $S = \frac{1}{2}$ and gives the characteristic epr signal (*Figure 5*).

Mössbauer spectra

The Mössbauer effect is a very useful tool to study the nature of the iron atoms in proteins[40, 41]. Since it is not a magnetic resonance technique it

can be observed equally well in paramagnetic (e.g. reduced ferredoxin) and non-magnetic (e.g. oxidized ferredoxin) materials. The Mössbauer effect can be used to measure the effective magnetic field produced at the nuclei by the electrons. Two parameters measured by Mössbauer spectroscopy are the chemical isomer shift (δ) which gives an idea of the valence and spin state of the iron atom and its degree of covalency, and the quadrupole splitting (ΔE) which is a probe of the local stereochemistry of the iron atom. Considerably more information regarding the valence and spin state of the iron can be obtained from the magnetic hyperfine spectra observed at low temperatures when the Mössbauer effect is studied in the presence and absence of an external magnetic field[42].

Since the Mössbauer effect in the case of iron is specific to ^{57}Fe nuclei and since the natural abundance of ^{57}Fe nuclei in iron is very low (about 2.2 per cent) it is advantageous to enrich the ferredoxins with ^{57}Fe before Mössbauer effect measurements. This can be easily achieved by chemical substitution in the case of ferredoxins without changing the conformation or biological activity of the protein. Enrichment with ^{57}Fe has also been achieved by growing *Euglena* on ^{57}Fe and then extracting the enriched ferredoxin[44]. The results were very similar to those from the chemically substituted ferredoxin.

The Mössbauer spectra of three plant ferredoxins in the reduced state at two different temperatures are shown in *Figure 6*. The spectrum of the

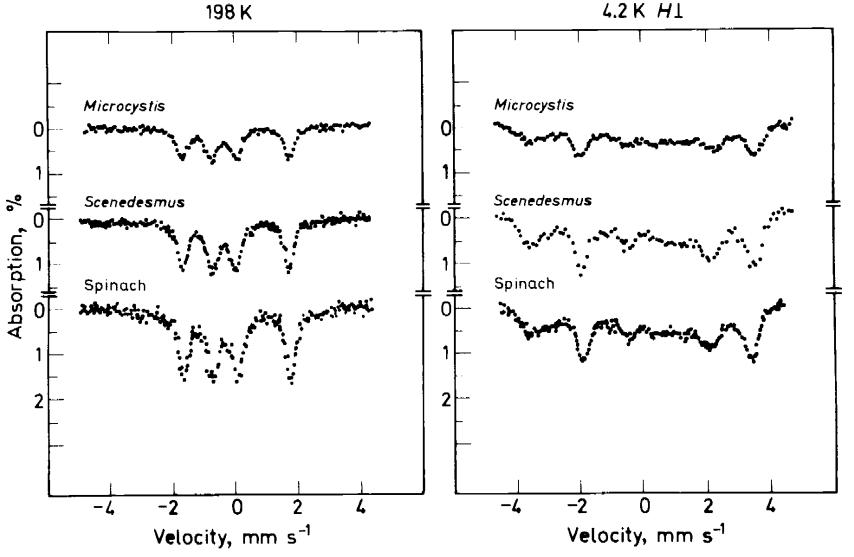


Figure 6. Mössbauer spectra of reduced ferredoxins from two algal and one plant species, at 198 K (left), and at 4.2 K (right) in a small field perpendicular to the γ-rays, illustrating the magnetic hyperfine splitting[92].

oxidized ferredoxins (not shown in the diagram) has two lines of almost equal width with a chemical shift of about $+0.20$ mm s^{-1} relative to

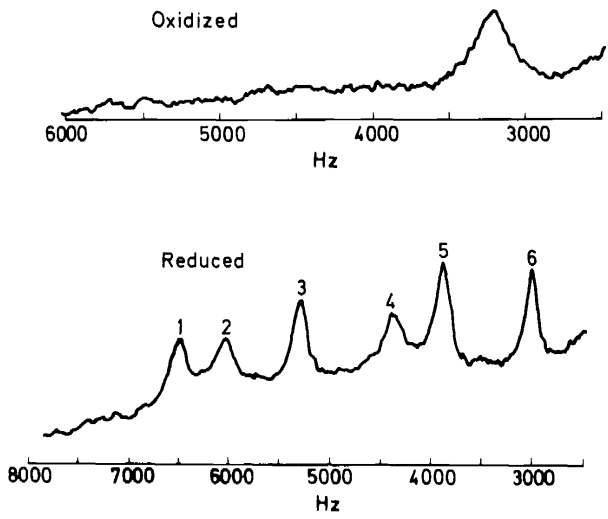
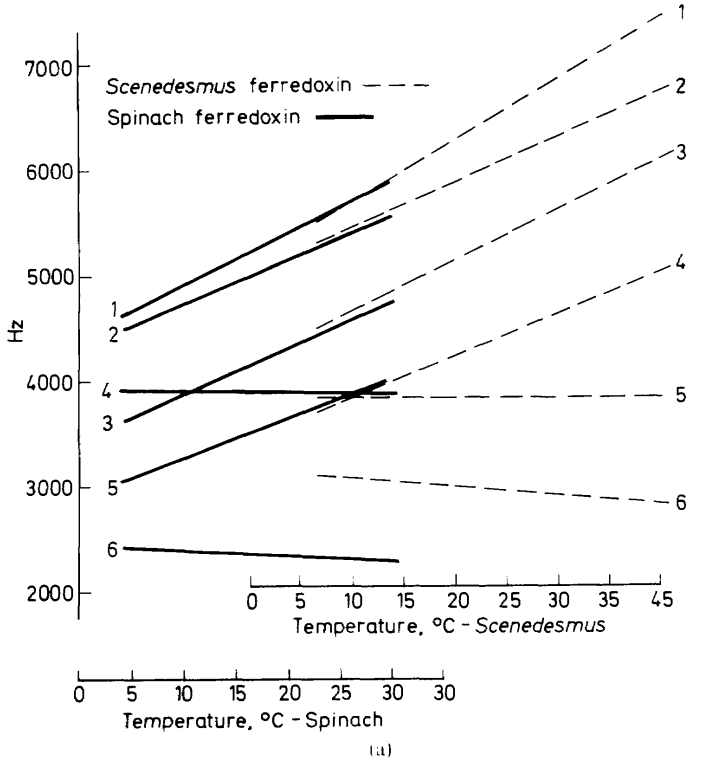


Figure 7. Low-field region of the pmr spectra of *Scenedesmus* ferredoxin, D_2O, 22°C. In the reduced form, the resonances due to six protons are visible and the peaks are numbered. (Unpublished results obtained in collaboration with W. D. Phillips and C. C. McDonald).

metallic iron and a quadrupole splitting of 0.60 mm s^{-1}. The spectrum is consistent with the high-spin Fe^{3+} atoms in almost equivalent states. The spectra of the reduced ferredoxins at high temperature (*Figure 6*) show four lines of almost equal width and depth. These are a superposition of two quadrupole split doublets; one doublet similar to that of the oxidized ferredoxin (Fe^{3+}) and the second with a larger chemical shift and quadrupole splitting which is characteristic of the high-spin Fe^{2+} atom.

The effect of a small magnetic field on the Mössbauer spectrum of reduced ferredoxin at very low temperatures showing magnetic hyperfine splitting is shown on the right hand side of *Figure 6*. The Mössbauer spectrum is split by the application of large magnetic fields, the effective field of the Fe^{3+} ions decreasing and that of the Fe^{2+} ions increasing in the applied field. The details of the interpretation of the Mössbauer spectra are given elsewhere[45]; suffice it to say that the resemblance of the spectra of ferredoxins from spinach, *Scenedesmus* and *Microcystis* again confirms the view that the active centres of all plant ferredoxins are identical in structure. Mössbauer studies have also been carried out on parsley, spinach and other ferredoxins by Dunham *et al.*[46] All these studies confirm the original model of Gibson[38], Thornley[39] and associates proposed for the active centre of ferredoxins (*Figure 5*).



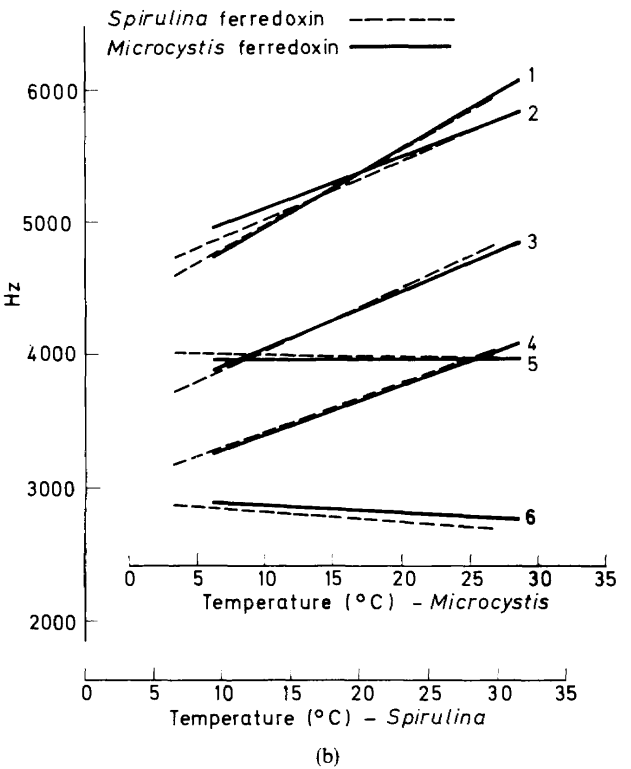


Figure 8. Temperature dependence of the low-field contact-shifted protons from reduced ferredoxins of (a) spinach and *Scenedesmus* and (b) *Spirulina* and *Microcystis* ferredoxins. The temperature scales have been offset to show the similarity of the patterns. Proton peaks are numbered as in *Figure 7*. (Unpublished results obtained in collaboration with W. D. Phillips and C. C. McDonald).

Proton magnetic resonance spectra

Phillips and his associates have studied the proton magnetic resonance (pmr) spectra of spinach, parsley, alfalfa, and soyabean ferredoxins[47, 48]. In order to measure the pmr spectra, the proteins were lyophilized, and dissolved under a nitrogen atmosphere in 0.2M Tris–DCl in 99.77 per cent D_2O, P_d 7.8. Contact shifted resonances were detected to low field in the spectra of both oxidized and reduced ferredoxins (for example, see *Figure 7*). The resonances are assigned to the β-CH_2 protons of the four cysteine residues that are thought to bind the iron–sulphur redox centre to the polypeptide chain. The temperature dependence of the contact shifts supported the proposal that the two iron atoms are antiferromagnetically coupled in both oxidized and reduced forms and the assignment for the valence states of high-spin $Fe^{3+} - Fe^{3+}$ and $Fe^{2+} - Fe^{3+}$ for the iron pairs in the oxidized and reduced ferredoxins, respectively. It seems that the temperature dependence of these contact shifts is very sensitive to protein structure. The whole pattern seems to be shifted at different temperatures in spinach with *Scenedesmus* (*Figure 8a*). The temperature dependent contact shifts of two blue-green algal ferredoxins are compared in *Figure 8b*. Once again these indicate that while there is variation between the algal and higher plant ferredoxins the blue-green algal ferredoxins are very similar. Interestingly in the contact shift spectrum of reduced soyabean ferredoxin preparations it was possible to detect the presence of two distinct genetic variants in approximately equal concentration[48]. Thus pmr studies, in addition to being a valuable tool for the study of the nature of the paramagnetic species, can also be used to detect minor variations in the amino acid sequences of ferredoxins prepared from the same species.

AMINO ACID SEQUENCES OF PLANT FERREDOXINS

The primary structures of ferredoxins from five species of plants are known[49]. There is remarkable similarity in the structures from that of *Scenedesmus* (green alga) to *Leuceana glauca* (tree). These proteins consist of 96 or 97 amino acid residues and all of them have alanine at their amino terminal end. About 60 per cent of the residues are invariant in the five sequences. Other prominent similarities are, (1) that five cysteine residues are found in identical positions in all the ferredoxins sequenced; there is no cysteine in position 18 in *Equisetum* ferredoxin[50] thus indicating that the other four invariant cysteines are involved in the binding of the two iron atoms in the active centre (*Figure 5*); (2) segments containing residues 18 to 26, and 35 to 50 and 62 to 66 are invariant except for a substitution of Ala in residue 42 of *Scenedesmus* ferredoxin instead of Ser in all other ferredoxins. Though the sequences of other ferredoxins are not known, from their amino acid compositions and spectral properties we may assume close similarities in the primary structures of ferredoxins from the blue-green algae to higher plants. The primary structure of *Scenedesmus* ferredoxin is compared with that of spinach ferredoxin in *Figure 9*.

Blue-green algae are considered to be the most primitive algae, ecologically related, and evolutionarily close, to the photosynthetic bacteria since they

```
   1                                                                  20
(A) Ala Thr Tyr Lys Val Thr Leu Lys Thr Pro Ser Gly Asp Gln Thr Ile Glu Cys Pro Asp
(B) Ala Ala Tyr Lys Val Thr Leu Val Thr Pro Thr Gly Asn Val Glu Phe Gln Cys Pro Asp

  21                                                                  40
(A) Asp Thr Tyr Ile Leu Asp Ala Ala Glu Glu Ala Gly Leu Asp Leu Pro Tyr Ser Cys Arg
(B) Asp Val Tyr Ile Leu Asp Ala Ala Glu Glu Glu Gly Ile Asp Leu Pro Tyr Ser Cys Arg

  41                                                                  60
(A) Ala Gly Ala Cys Ser Ser Cys Ala Gly Lys Val Glu Ala Gly Thr Val Asp Gln Ser Asp
(B) Ala Gly Ser Cys Ser Ser Cys Ala Gly Lys Leu Lys Thr Gly Ser Leu Asn Gln Asp Asp

  61                                                                  80
(A) Gln Ser Phe Leu Asp Asp Ser Gln Met Asp Gly Gly Phe Val Leu Thr Cys Val Ala Tyr
(B) Gln Ser Phe Leu Asp Asp Asp Gln Ile Asp Glu Gly Trp Val Leu Thr Cys Ala Ala Tyr

  81                                                                96
(A) Pro Thr Ser Asp Cys Thr Ile Ala Thr His Lys Glu Glu Asp Leu Phe
(B) Pro Val Ser Asp Val Thr Ile Glu Thr His Lys Glu Glu Glu Leu Thr Ala
```

—— = Invariant amino acids in all 5 sequenced plant ferredoxins (60 in all).
(A) Scenedesmus[14] (B) Spinach[94]

Figure 9. Sequences of plant ferredoxins.

do not have their photosynthetic pigments localized in chloroplasts and they are prokaryotes. Some blue-green algae like *Spirulina sp.* are essentially indistinguishable from some of the cyanophytes of microflora represented in the Bitter Springs (Australia) fossils believed to be about 900 million years of age[51]. But, like the green algae and higher plants (and unlike the photosynthetic bacteria), the blue-green algae liberate oxygen during photosynthesis and ferredoxin is an essential catalyst in the overall reaction. The amino acid composition and the nature of the active centre of ferredoxins from all species ranging from 'blue-greens' to higher plants are similar. It is probable that the development of the oxygen-evolving system and the concomitant development of plant-type ferredoxin marked the final stage in the evolution of the ferredoxin molecule from those of primitive bacteria (see later). The similarity of the ferredoxins in blue-green alga and in the chloroplasts of higher plants strongly supports the idea that chloroplasts are derived from the symbiotic association between a blue-green alga and a non-photosynthetic eukaryotic cell[52, 53].

BACTERIAL AND MAMMALIAN FERREDOXINS CONTAINING TWO IRON ATOMS

Iron–sulphur proteins resembling plant ferredoxins have been isolated from various bacterial and mammalian sources (*Table 1*). The active centre of these proteins containing two atoms of iron is almost identical to that of plant ferredoxins[28, 45, 46, 54]. Of the former the primary structure of adrenodoxin[55], from mammalian adrenal 11β steroid hydroxylase system, and of putidaredoxin[56], a component of the camphor hydroxylase system of *Pseudomonas putida*, have been studied in detail. Putidaredoxin has 114 and

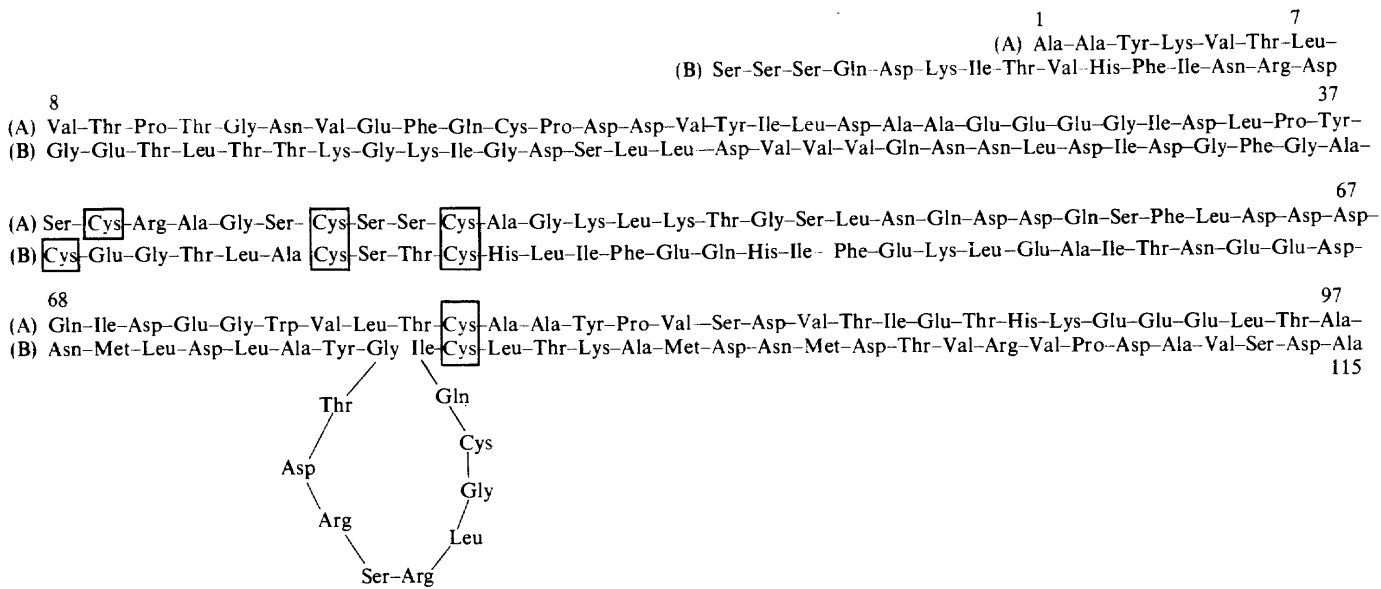
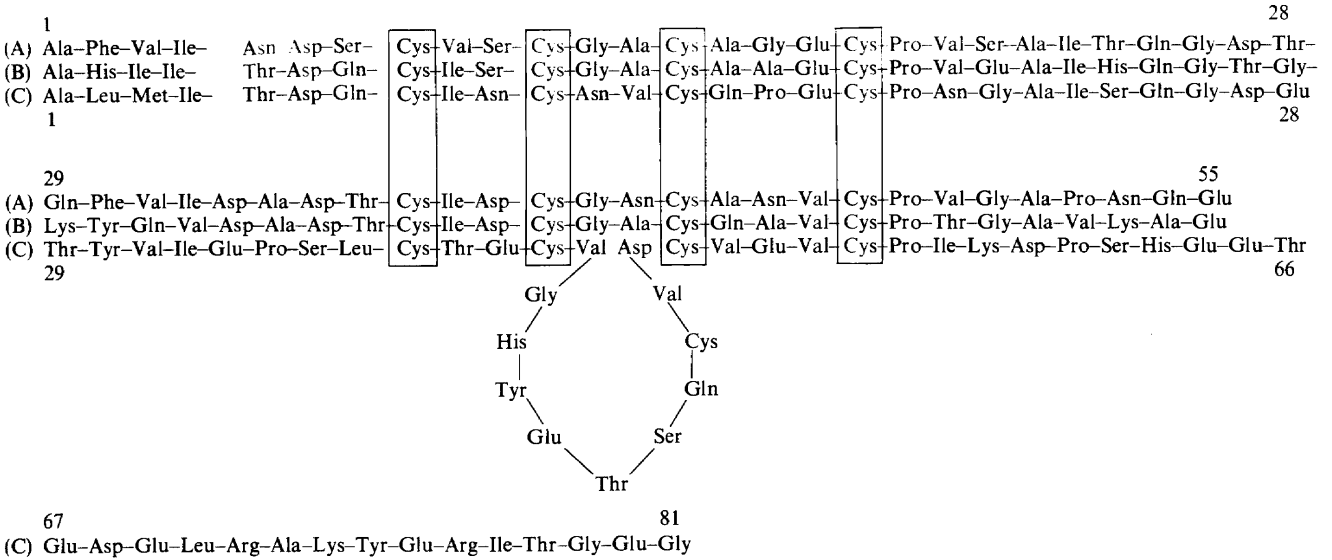


Figure 10. Comparison of spinach ferredoxin and adrenodoxin sequences.
(A) Spinach ferredoxin[94], (B) Bovine adrenodoxin[55]



(A) *C. butyricum*:[107] (B) *C. tartarivorum*:[61] (C) *Chromatium*:[62]

Figure 11. Comparison of sequences of *Clostridium* and *Chromatium* ferredoxins

adrenodoxin 115 amino acid residues. In common with plant ferredoxins they contain at least one –Cys–x–x–Cys– grouping in their sequence. Tsai *et al.*[56] have identified a number of homologous segments in the primary structures of putidaredoxin and adrenodoxin and the sequences of these two ferredoxins exhibit a fair degree of similarity to the sequences of plant ferredoxins. We have aligned the sequences of adrenodoxin and spinach ferredoxin in *Figure 10* to point out some of the similarities. A statistical analysis of various ferredoxin sequences by Barker *et al.*[57] confirms the relationship between adrenodoxin and plant ferredoxins which suggests that all these ferredoxins could have evolved from a common ancestral gene. However, in spite of the identity of their active centre and similarity in their amino acid sequence, the two-iron ferredoxins of one type are not completely interchangeable with the two-iron ferredoxins of another type in their biological function. This could possibly be due to differences in their tertiary structures or due to differences in the structures of various reductases with which they combine during physiological electron transfer processes.

FERREDOXINS CONTAINING EIGHT IRON ATOMS

Ferredoxins containing eight atoms of iron have so far been obtained only from bacteria, both photosynthetic and non-photosynthetic. The optical absorption of these ferredoxins are characterized by a maximum at 390 nm and they differ in their biological action from the plant ferredoxins in that they transfer two electrons at a time. All these ferredoxins, thus far analyzed, except *Chromatium* ferredoxin, consist of about 55 amino acid residues with a molecular weight of approximately 6000 daltons. As in the plant ferredoxins the iron and 'labile sulphur' of these proteins can be removed by the action of reagents like trichloracetic acid and mercurials and the original ferredoxin can be reconstituted from the apoprotein by the addition of iron salt and sulphide in a reducing medium[58]. The structure of the iron–sulphur complex of this type of ferredoxin is not as well established as the plant ferredoxins. Magnetic susceptibility measurements have indicated strong antiferromagnetic exchange coupling between the component iron atoms in *C. pasteurianum* ferredoxin[59]. An x-ray investigation of crystalline *M. aerogenes* ferredoxin has shown that the eight iron and sulphur atoms exist as two identical clusters of 4Fe plus 4S atoms[60]. However, we still do not have enough information to correlate the magnetic resonance properties of these proteins and their electron transferring capacity.

The amino acid sequences of five ferredoxins from obligate fermentative bacteria are known[61]. In addition to the invariant positions of eight cysteine residues in the sequences of all these five ferredoxins there are many identical segments in their structures confirming the homologous nature of these proteins. The primary structure of *Chromatium* ferredoxin contains an extra 26 amino acid residues[62]. However, the similarity of this molecule to the other bacterial ferredoxins in this group is borne out by the invariant positions of many amino acid residues when its primary structure is realigned, as was done by Matsubara *et al.*[63], and then compared with the clostridial ferredoxins. A similar comparison of *Chromatium* ferredoxin with two clostridial ferredoxins is depicted in *Figure 11*.

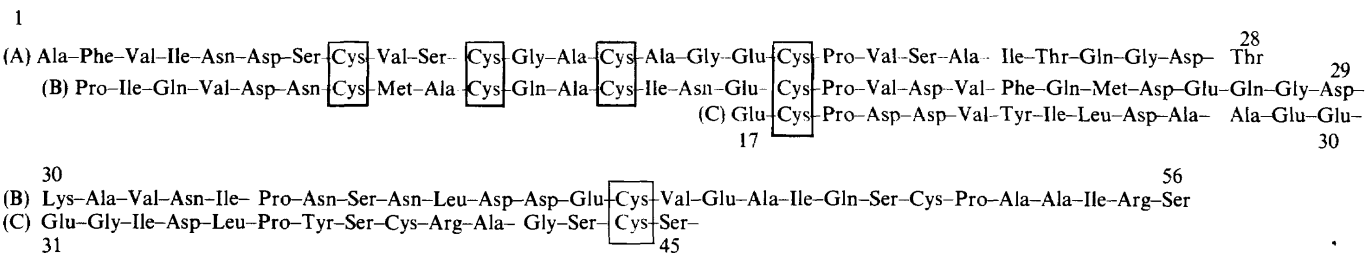


Figure 12. Sequence of *D. gigas* ferredoxin[66] (B) compared with analogous segments of the ferredoxins of *C. butyricum*[107] (A) and spinach[94] (C).

THE FERREDOXINS CONTAINING FOUR IRON ATOMS

Three ferredoxins containing four atoms each of iron and sulphur per molecule are fairly well characterized (*Table 1*). One of these, *Chromatium* high potential iron protein (HIPIP) is quite distinct in its properties from other ferredoxins[64, 65]. No biological function has yet been found for this protein. The ferredoxin from the sulphate reducer *Desulphovibrio gigas*[66] is interesting due to the fact that the primary structure of this protein is homologous in parts to segments of *Clostridia* ferredoxins and is similar in other parts to segments of plant ferredoxins (T. H. Jukes, personal communication). Their primary structures are compared in *Figure 12*. *Bacillus polymyxa* ferredoxin[67] has a molecular weight of 9000 and on reduction accepts one electron per molecule[68]. These four-iron ferredoxins may be evolutionary links between the eight- and the two-iron ferredoxins and so are being actively studied by various groups.

ARE FERREDOXINS PRIMITIVE PROTEINS?

Ever since Oparin enunciated his theory about the abiogenic origin of life on earth attempts have been made to synthesize in the laboratory compounds like amino acids, purines, pyrimidines etc. under conditions which simulated prebiotic environments[69]. There is also a vigorous search to detect the presence of precursors of biological molecules like sugars, proteins and nucleic acids in extra-terrestrial matter[70–72]. The presence of these precursors in meteorites and other planets would indirectly prove their existence in the prebiotic earth's atmosphere. Amino acids like glycine, alanine, valine, glutamic acid, aspartic acid, proline, serine, cysteine and isoleucine have been synthesized under simulated primitive earth conditions[49]. Analysis of the Murchison and Murray meteorites and moon samples collected in the Apollo missions have shown the occurrence of the first six of the above-mentioned amino acids in these bodies. It is interesting that 64 per cent of *C. butyricum* ferredoxin is made up of these six amino acids. The clostridial ferredoxins are relatively small proteins and the iron and sulphur which form the active centre of these proteins can be added to the apoprotein by a non-enzymic process. They have a very low redox potential close to that of hydrogen gas. All these properties suggest the possibility that a ferredoxin-like protein would have functioned even in very primitive organisms.

FERREDOXINS AS A TOOL IN PHYLOGENETIC CLASSIFICATION

It is now a generally accepted thesis that any mutation in the nucleotide codons of the gene directing the synthesis of a particular protein will be expressed by an amino acid substitution in the sequence of the protein, i.e. the primary structures of homologous proteins are reflections of the nucleotide sequences of their genetic material. The ferredoxins are found in all species of organisms. The amino acid sequences of ferredoxins from five species of anaerobic bacteria are known and they show a high degree of homology. Also, the positions of certain key amino acids like the amino terminal alanine residue and the eight cysteine residues, which are thought to chelate the iron atoms, are identical in all these five ferredoxins. Thus these

ferredoxins would probably have evolved from a common ancestral gene. The plant ferredoxins are about double the length of clostridial ferredoxins but the two groups show certain similarities in their properties and amino acid sequences. A quantitative comparison of the amino acid sequences of plant and *Clostridia* ferredoxins has indicated evolutionary relationships between these two groups but the evolutionary distances may be large[57, 63]. The ferredoxin from the red photosynthetic bacterium *Chromatium* is intermediate in length between *Clostridia* and plant ferredoxins but in its sequence and function it is closer to the *Clostridia* type than to the plant ferredoxins. We do not yet know the complete sequence of a ferredoxin from a green photosynthetic bacteria. However, from the known amino acid composition and terminal residues of ferredoxins from this latter group we could predict that they are intermediate between the non-photosynthetic anaerobes and the red *Chromatium*[73–75]. The position of the sulphate reducer *D. gigas* is based on a comparison of the amino acid sequence of its ferredoxin with bacterial and plant ferredoxins (*Figure 12*) and on the suggestion of Schlegel[75] from metabolic considerations that sulphate reducing bacteria can be placed between the anaerobic photolithotrophs (e.g. *Chromatium*) and the blue-green algae in the evolutionary ladder. Thus from a comparative study of the primary structure and function of ferredoxins from various species we could tentatively propose a phylogenic classification as outlined in *Figure 13*.

Anaerobic bacteria → Green photosynthetic bacteria → Red (sulphur) photosynthetic bacteria →

Sulphate reducing bacteria (Desulphovibrio) → Algae → Higher plants
Algae → Animals

Figure 13. Evolutionary development of ferredoxins[49].

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