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Comparative Studies on Two Ferredoxins from the Cyanobacterium *Nostoc* Strain MAC

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Two ferredoxins were isolated from the cyanobacterium *Nostoc* strain MAC grown autotrophically in the light or heterotrophically in the dark. In either case approximately three times as much ferredoxin I as ferredoxin II was obtained. Both ferredoxins had absorption maxima at 276, 282 (shoulder), 330, 423 and 465 nm in the oxidized state, and each possessed a single 2Fe–2S active centre. Their isoelectric points were approx. 3.2. The midpoint redox potentials of the ferredoxins differed markedly; that of ferredoxin I was −350 mV and that of ferredoxin II was −455 mV, at pH 8.0. The midpoint potential of ferredoxin II was unusual in being pH-dependent. Ferredoxin I was most active in supporting $NADP^+$ photoreduction by chloroplasts, whereas ferredoxin II was somewhat more active in pyruvate decarboxylation by the phosphoroclastic system of *Clostridium pasteurianum*. Though the molecular weights of the ferredoxins determined by ultracentrifugation were the same within experimental error, the amino acid compositions showed marked differences. The *N*-terminal amino acid sequences of ferredoxins I and II were determined by means of an automatic sequencer. There are 11–12 differences between the sequences of the first 32 residues. It appears that the two ferredoxins have evolved separately to fulfil different roles in the organism.

Ferredoxins from cyanobacteria (blue–green algae) appear to be of the type characteristic of higher plants, having a mol.wt. of about 11000, possessing a single 2Fe–2S active centre and transferring one electron (Buchanan & Arnon, 1970; Hall *et al.*, 1975*b*; Palmer, 1975). Since cyanobacteria represent an intermediate stage in evolution between the anaerobic photosynthetic bacteria and green plants, study of their ferredoxins might give some insight into the biochemical evolution of photosynthesis. The properties of the ferredoxins from a number of cyanobacteria have been summarized (Andrew *et al.*, 1975; Hall *et al.*, 1975*a*).

A few cyanobacteria such as *Aphanocapsa* 6714, *Chlorogloeopsis* (*Chlorogloea*) *fritschii* and *Nostoc* strain MAC are capable of growth both autotrophically in the light and heterotrophically in the dark (Hoare *et al.*, 1971). The ferredoxins present in cells grown under these different conditions are being isolated in the Department of Biochemistry, University College of Wales, Aberystwyth and their properties studied. We now report a study of two ferredoxins from *Nostoc* strain MAC, a filamentous cyanobacterium of the order Nostocales, originally isolated from negatively geotropic coralloid roots of the cycad *Macrozamia lucida* L. Johnson (Bowyer & Skerman, 1968). A preliminary report has been given of some of this work (Hutson & Rogers, 1975).

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The presence of two ferredoxins in *Nostoc* strain MAC was unexpected. Although two molecular species had been found in a facultative photosynthetic bacterium *Rhodospirillum rubrum* (Shanmugam *et al.*, 1972; Yoch *et al.*, 1975), a nitrogen-fixing obligate aerobic bacterium *Azotobacter vinelandii* (Yoch & Arnon, 1972), a nitrogen-fixing bacterium *Bacillus polymyxa* (Stombaugh *et al.*, 1973) and, detected by p.m.r.-spectral studies, in the higher plant *Glycine max* (Glickson *et al.*, 1971), there had at that time (Hutson & Rogers, 1975) been no report of two ferredoxins in a cyanobacterium. However, two ferredoxins have now been reported to occur also in *Aphanothece sacrum* (Hase *et al.*, 1975), *Spirulina maxima* (Cammack *et al.*, 1977*b*), *Nostoc verrucosum* (Shin *et al.*, 1977) and *Nostoc muscorum* (Hase *et al.*, 1976*b*). There have also been reports of two ferredoxins in higher plants; in the hybrid *Nicotiana glutinosa* × *N. glauca* (Kwanyuen & Wildman, 1975) and in *Pisum sativum* (Mukhin *et al.*, 1975), *Phytolacca americana* (Suzuki, 1976) and *Equisetum telmateia* (Hase *et al.*, 1977*a*). Some sequence heterogeneity

occurs in the ferredoxins from different individual trees of *Lucaena glauca* (Benson & Yasunobu, 1969). The two ferredoxins in *Nostoc* strain MAC and some other cyanobacteria have probably been inherited by the corresponding genes from an ancestral organism, probably after gene duplication (Hase *et al.*, 1975, 1977*b*).

Materials and Methods

Materials

Sephadex chromatographic resins were obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose (Whatman DE52) was obtained from W. and R. Balston, Maidstone, Kent, U.K. Acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylenediamine, *NN'*-dimethyl-*p*-phenylenediamine, *o*-phenanthroline and deoxyribonuclease were from BDH Chemicals, Poole, Dorset, U.K. Ribonuclease, bovine serum albumin and *p*-hydroxymercuribenzoate were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Coomassie Brilliant Blue was obtained from E. Gurr, London S.W.6, U.K. Ampholine (pH 2.5–6) was purchased from LKB Produkter, Stockholm, Sweden. Except where noted other chemicals were from Boehringer Corp., Lewes, Sussex, U.K.

Organism and growth conditions

Nostoc strain MAC was grown both autotrophically in light and heterotrophically in the dark (Hoare *et al.*, 1971), on medium Cg10 of Van Baalen (1968) supplemented with 0.9 g of $KHCO_3$/litre for autotrophic growth, or 1.8 g of glucose/litre for heterotrophic growth. In the former case cultures at 30°C were gassed continuously with air/CO_2 (19:1) during illumination (5000 lx). The cyanobacteria were grown in 10-litre flasks with a 6% (v/v) inoculum. The yield from eight flasks was typically 140 g of cell paste, which was resuspended in 200 ml of 10 mM-Tris/HCl, pH 7.8.

Extraction and purification

Cell suspensions were added slowly with vigorous stirring to 1.5 litres of acetone at −20°C. This and all subsequent stages of the purification were carried out in a cold-room at 4°C. The resulting acetone-dried powder (about 30 g yield) was homogenized in 300 ml of 0.15 M-Tris/HCl, pH 7.8, and the suspension left for approx. 15 h after addition of 5 mg each of deoxyribonuclease and ribonuclease to degrade polynucleotides. The mixture was centrifuged at 40000 *g* for 60 min and the supernatant was adjusted to 60% saturation with $(NH_4)_2SO_4$. Anion-exchange chromatography was carried out on a column (65 cm × 2.5 cm) of DEAE-cellulose (DE52). The resin was equilibrated with 0.15 M-Tris/HCl buffer, pH 7.8, and the supernatant obtained by centrifugation at 40000 *g* for 60 min after $(NH_4)_2SO_4$ treatment was dialysed for 24 h against the same buffer before it was applied to the column. The column was washed with 500 ml of 0.15 M-Tris/HCl, pH 7.8, next with 500 ml of the same buffer containing 0.1 M-NaCl, and then developed with 0.15 M-Tris/HCl, pH 7.8, containing 0.2 M-NaCl.

Under these conditions of chromatography two reddish-brown zones became evident on the column. Development was continued until the faster-moving ferredoxin, designated ferredoxin II, and the more tightly bound ferredoxin, designated ferredoxin I, were completely eluted from the column. Elution volumes were approx. 1000 ml and 1500 ml respectively. Fractions of each ferredoxin with A_{423}/A_{276} ratios greater than 0.5 were pooled and separately concentrated by using small (6 cm × 1 cm) DEAE-cellulose columns.

Studies of homogeneity by analytical polyacrylamide-gel electrophoresis indicated that in most preparations ferredoxin I needed no further purification. However, appreciable absorbance at 260 nm and a consequent low A_{423}/A_{276} ratio indicated that ferredoxin II preparations were frequently contaminated by polynucleotide material, and further purification was necessary. This was done by gel filtration on a column (60 cm × 2.5 cm) of Sephadex G-50 developed with 0.15 M-Tris/HCl, pH 7.8, containing 0.2 M-NaCl. A similar procedure was also occasionally necessary for the ferredoxin I fraction. The ferredoxin fractions with A_{423}/A_{276} ratios greater than 0.5 were pooled and concentrated by using a small DEAE-cellulose column before storage at −20°C or immediate use.

Typical yields from 30 g of acetone-dried *Nostoc* strain MAC powder prepared from photoautotrophically or heterotrophically grown cells were 35 mg of ferredoxin I and 10 mg of ferredoxin II. Ferredoxin yields from acetone-dried powders were about twice as much as from the equivalent weight of cell paste subjected to standard French-pressure-cell treatment.

Analytical polyacrylamide-gel electrophoresis and isoelectric focusing

The disc electrophoresis technique (Davis, 1964) was used, without spacer or sample gels. Electrophoresis was carried out at pH 8.0 on 10%, 15% and 25% (w/v) acrylamide gels; 3.6% of the total acrylamide was *NN'*-methylenebisacrylamide. For detection of protein components in the gels, staining with Coomassie Blue or Amido Black was used (Andrew *et al.*, 1975).

Isoelectric focusing was carried out on gel slabs containing 1.5% (v/v) Ampholine (pH 2.5–6) by

using an LKB Multiphor apparatus (LKB Instruments, Rockville, MO, U.S.A.). Preparation of the gels and conditions of electrophoresis were as described in the manufacturers' manual. The pH gradient along the gel was determined directly with a digital pH-meter by using a surface electrode.

Amino acid analysis and sequence studies

Samples were hydrolysed at 110°C for 24 or 72h in 6M-HCl in O_2-free sealed ampoules, and analyses were performed on a Locarte single-column autoanalyser at the Macromolecular Analysis Centre, University of Birmingham. Quantification of the amino acids was through a Nova 1220 computer on-line to the analyser, with corrections for loss of threonine and serine during hydrolysis. Tryptophan was determined by a spectrophotometric method (Gaitonde & Dovey, 1970), and cysteine by titration with 5,5′-dithiobis-(2-nitrobenzoic acid) (Chung *et al.*, 1971).

N-Terminal amino acid sequences of the ferredoxins were determined by means of a sequenator (Edman & Begg, 1967). Automated sequence analysis of carboxymethylated protein was carried out on a Beckman 890C protein sequencer by using the 'fast protein' program supplied by the manufacturers (Beckman program no. 072172 C). Phenylthiohydantoin derivatives were identified as described by Haslett & Boulter (1976).

Midpoint redox potentials

Midpoint redox potentials of the ferredoxins were determined by the general method used for other ferredoxins (Cammack *et al.*, 1977*b*) and xanthine oxidase (Cammack *et al.*, 1976). The proteins were adjusted to various potentials by adding $Na_2S_2O_4$ or $K_3Fe(CN)_6$ in the presence of dye mediators and a platinum electrode. The pH was monitored with a glass electrode. Samples were taken and frozen for measurement of the e.p.r. signal (g_y) at $g = 1.96$ corresponding to reduced ferredoxin. The midpoint potentials were determined from plots of the intensity of this signal versus applied redox potential. The e.p.r. spectra of the ferredoxins have been presented elsewhere (Cammack *et al.*, 1977*b*).

Other analyses

Non-haem iron content was determined as described by Harvey *et al.* (1955) by titration with *o*-phenanthroline. Labile sulphur was determined by titration with *NN*′-dimethyl-*p*-phenylenediamine (Kimura & Suzuki, 1967).

Total sulphur was found by titration with *p*-hydroxymercuribenzoate (Boyer, 1954), enabling cysteine sulphur to be deduced once labile sulphur was determined.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Molecular-weight determinations

Short-column sedimentation-equilibrium experiments were performed by the methods of Van Holde & Baldwin (1958) or Yphantis (1964) at 20°C with ferredoxin solutions in 0.15M-Tris/HCl, pH 7.8, in a Beckman-Spinco model E ultracentrifuge equipped with Rayleigh interference optics. Experiments by the method of Van Holde & Baldwin (1958) were performed at a speed of 20410rev./min, at an initial protein concentration of approx. 2mg/ml. For the Yphantis (1964) method, rotor speed was 44770rev./min, and the initial protein concentration was 0.5mg/ml. For both types of experiment a 10mm double-sector cell was used with ferredoxin solution in one compartment and buffer (diffusate) in the other. The ferredoxins were quite stable for the duration (24–36h) of the experiments.

The partial specific volumes ($\bar{v}$) were calculated from amino acid composition by the method of Cohn & Edsall (1943).

Activity determinations

The photosynthetic activity of the ferredoxins was determined by $NADP^+$ photoreduction by using barley chloroplasts depleted of native ferredoxin (Delaney *et al.*, 1977).

For assays each 1cm-light-path cuvette contained (in μmol): Tris/HCl (pH 8), 45; NaCl, 60; $MgCl_2$, 12; KH_2PO_4, 5; $NADP^+$, 0.49; the required amount of ferredoxin; and chloroplast suspension equivalent to approx. 30μg of chlorophyll; total volume 3.0ml. Photoreduction of $NADP^+$ was followed by the increase in A_{340} on illumination with saturating (140000lx) light, after 30s and 1min illumination. Over this time interval reduction of $NADP^+$ proceeded linearly with time. Total chlorophyll was determined as described by Arnon (1949).

Activity of the ferredoxins in the pyruvate phosphoroclastic reaction was measured by using extracts from *Clostridium pasteurianum* depleted of native ferredoxin by passage through a column (8cm × 3cm) of DEAE-cellulose equilibrated with 0.15M-Tris/HCl (pH 7.8). For assays each 1cm-light-path cuvette contained (in μmol): NaH_2PO_4 (pH 6.5), 50; CoA, 0.13; sodium pyruvate, 10; the required amount of ferredoxin; and extract of *C. pasteurianum* containing approx. 5mg of protein; total volume 1.0ml. The acetyl phosphate formed after 15min incubation at 30°C was determined as acetohydroxamate by formation of a complex with $FeCl_3$ and measuring the A_{540} (Mortenson *et al.*, 1962).

Ferredoxin from *C. pasteurianum* was prepared as described by Mortenson *et al.* (1962) and used as a reference ferredoxin in activity determinations.

Results and Discussion

A typical elution profile for the separation of the two *Nostoc* strain MAC ferredoxins on DEAE-cellulose chromatography has been given in Fig. 1a of Hutber *et al.* (1977) (ferredoxins I and II in that legend should be transposed). About twice as much ferredoxin I as ferredoxin II is present in cell extracts (Hutber *et al.*, 1977), but because of greater loss of ferredoxin II during subsequent purification stages yields of pure ferredoxin I were 3–4 times those of ferredoxin II.

In each case with the cyanobacteria possessing two ferredoxins, that present in greatest quantity (ferredoxin I in *N. verrucosum*, *Nostoc* strain MAC and *S. maxima*) is eluted more slowly from DEAE-cellulose columns. However, the ratio of the two ferredoxins (I/II) varies quite considerably, from 5:1 for *A. sacrum* (Hase *et al.*, 1975), and possibly greater for *N. muscorum* (Hase *et al.*, 1976*b*) and *S. maxima* (Cammack *et al.*, 1977*b*), to nearly equal quantities in *N. verrucosum* (Shin *et al.*, 1977).

Homogeneity of the protein fractions

Samples of both ferredoxins isolated from *Nostoc* strain MAC were subjected separately and in mixture to analytical electrophoresis, which in all cases revealed a single heavily stained band with no minor components evident; in 10 and 15% (w/v) gels the ferredoxins migrated at the anion front. The two ferredoxins were therefore not separable under these conditions, indicating close similarity in their molecular weights.

The two ferredoxins were also subjected separately and in mixture to isoelectric focusing on slabs of polyacrylamide gel, by using an Ampholine range of pH 2.5–6.0. Where ferredoxins were run separately a single precipitated band of material, with no trace of minor components, was seen. The pI values for ferredoxins I and II were sufficiently different for two bands to be seen when mixtures were run. The reported values of approx. 3.2 are significantly lower than values of 3.7 suggested by less rigorous methods for *Anabaena flos-aquae* (Andrew *et al.*, 1975) and *C. pasteurianum* (Lovenberg *et al.*, 1963) ferredoxins, and of approx. 4.0 for the *B. polymyxa* ferredoxins (Stombaugh *et al.*, 1973).

Composition and physical properties

The other properties of the two ferredoxins from *Nostoc* strain MAC are summarized in Table 1. Unlike the absorption spectra of the *N. verrucosum* ferredoxins, which slightly differed (Hase *et al.*, 1976*b*), the absorption spectra were identical, and are similar to those of other plant-type ferredoxins. The A_{423}/A_{276} ratio is dependent on the aromatic amino acid content of the particular ferredoxin, though the absorption of the chromophore also contributes to the u.v. absorption. The similarity in each case of the A_{423}/A_{276} ratio compared with that for other ferredoxins lacking tryptophan, together with the absence of a shoulder at approx. 290 nm, therefore indicated the likelihood that tryptophan was absent from both ferredoxins. On reduction with dithionite the absorption in the visible region was considerably diminished. The molar absorption coefficients at 423 nm were estimated on the basis of both protein and iron determinations. Provisionally assigned values of 6.5×10^3 and 6.1×10^3 litre·mol^{-1}·cm^{-1} for ferredoxin I and ferredoxin II respectively are in good agreement with those reported for the ferredoxins from *Anacystis nidulans* (Yamanaka *et al.*, 1969) and *Nostoc* sp. (Mitsui & Arnon, 1971). However, these values are appreciably lower than the 9.6×10^3–9.7×10^3 litre·mol^{-1}·cm^{-1} quoted for the ferredoxins from spinach (Tagawa & Arnon, 1968) and *Phormidium foveolarum* (Mitsui & San Pietro, 1973), and of 10.4×10^3–11.5×10^3 litre·mol^{-1}·cm^{-1} quoted for the *A. sacrum* and *N. verrucosum* ferredoxins (Wada *et al.*, 1974; Hase *et al.*, 1975; Shin *et al.*, 1977).

Analyses of non-haem iron and labile sulphur were made on a number of different preparations of ferredoxins I and II. They left no doubt that both ferredoxins possessed 2 atoms of iron and 2 atoms of labile sulphur per molecule, in common with all cyanobacterial ferredoxins so far studied. The molecular weights of the ferredoxins were determined by meniscus-depletion and low-speed equilibrium sedimentation. Experimental plots of r^2 (r is distance from the rotor) versus log c (log of concentration of the protein, expressed as fringe displacements) for the meniscus-depletion method, and of $(1/r)(\partial c/\partial r)$ versus c for the low-speed method, were linear, confirming the homogeneity of the ferredoxins. The partial specific volumes calculated from amino acid composition were 0.715 and the average molecular weights determined for ferredoxins I and II were approx. 12000. These values are somewhat higher than those determined from amino acid composition (Table 1). This may reflect uncertainty in the value of partial specific volume taken from amino acid composition compared with that for the holoprotein, an error of 1% in $\bar{v}$ giving an approx. 3% error in the molecular weight. The molecular weights of iron–sulphur proteins determined from sequencing data have often been 10% or more different from the hydrodynamic value (Orme-Johnson, 1973). Clearly, though, the molecular weight of these ferredoxins is the same as that of many other cyanobacterial and algal ferredoxins (Andrew *et al.*, 1975, 1976), includ-

Table 1. *Properties of the ferredoxins from Nostoc strain MAC*
Some values are given as means ± S.D. for *n* experiments. Activity is given as decimal fraction of that of *C. pasteurianum* ferredoxin.

	Ferredoxin I	Ferredoxin II
Absorption maxima ...	276, 282 (shoulder), 330, 423, 465	276, 282 (shoulder), 330, 423, 465
Ratio $A_{max.}/A_{276}$ ...	1.00, —, 0.64, 0.52, 0.50	1.0, —, 0.70, 0.50, 0.46
Non-haem Fe (atoms/molecule)	1.95 ± 0.13, $n = 12$	1.98 ± 0.16, $n = 10$
Labile sulphur (atoms/molecule)	1.90 ± 0.21, $n = 10$	1.88 ± 0.29, $n = 10$
Total sulphur (atoms/molecule)	7*	6–7*
	7†	6†
pI	3.2	3.1
Molecular weight		
By ultracentrifugation	12050 ± 700, $n = 3$	12250 ± 650, $n = 5$
From amino acid composition	10850	10500
Activity (cf. *C. pasteurianum* ferredoxin)		
$NADP^+$ photoreduction	0.76	1.38
Phosphoroclastic reaction	0.84	0.66
Midpoint redox potential (mV) at pH 8.0‡	−350	−455

* By titration with *p*-hydroxymercuribenzoate.
† From ΔA_{250} after reaction with excess of *p*-hydroxymercuribenzoate.
‡ Values from Cammack *et al.* (1977*b*).

Table 2. *Amino acid composition of Nostoc strain MAC ferredoxins I and II*
Values, based on ten analyses, are given as the nearest integer for the minimum molecular weight.

	Content (residues/molecule)				
	Ferredoxin			Ferredoxin	
	I	II		I	II
Asx	13	15	Met	Not present	
Thr	10	6	Ile	6	6
Ser	7	7	Leu	8	7
Glx	13	12	Phe	1	3
Pro	3	3	Lys	5	3
Gly	6	7	Tyr	5	3
Ala	6	10	His	1	1
Val	8	6	Arg	1	3
½CyS*	5	4	Trp†	Not present	

* From titration with *p*-hydroxymercuribenzoate.
† Determined by a spectrophotometric method.

ing the major ferredoxin in *N. muscorum* (Hase *et al.*, 1976*b*). In contrast, the two ferredoxins in *N. verrucosum* (Shin *et al.*, 1977) are quoted as having a mol.wt. of approx. 18000.

Although in all the properties so far discussed the two ferredoxins were indistinguishable, they differed markedly in amino acid composition (Table 2). The analyses are based on 24h hydrolyses with corrections made for losses of serine and threonine; valine and isoleucine were confirmed by analyses at longer hydrolysis times. In particular ferredoxin I contained appreciably more threonine, valine, lysine and tyrosine, whereas ferredoxin II contained more alanine, phenylalanine and arginine. Neither methionine nor tryptophan, the latter determined spectrophotometrically, were present. Both are usually absent from cyanobacterial and algal ferredoxins, though they are apparently present in the minor ferredoxin of *A. sacrum* (Hase *et al.*, 1975). Both compositions differed significantly from that of *N. muscorum* major ferredoxin (Hase *et al.*, 1976*b*); in particular the latter possesses two histidine residues, unlike the other cyanobacterial ferredoxins so far studied.

Samples were not oxidized before analysis and cysteine had therefore to be determined separately in the native protein by titration with *p*-hydroxymercuribenzoate. For ferredoxin I, 9mol of reagent reacted with each mol of ferredoxin (Table 1), indicating the likelihood that five cysteine residues are present, since the two labile sulphide groups would each react with 2 molecules of reagent. Results for ferredoxin II were less conclusive, but suggest that in this case only four cysteine residues are present. Most plant-type ferredoxins possess either five or six cysteine residues; exceptions include the ferredoxin from the alga *Bumelleriopsis* (Böger, 1970) and the major ferredoxin of the cyanobacterium *N. muscorum* (Hase *et al.*, 1976*b*). A report of only four cysteine residues in the major ferredoxin of *A. sacrum* (Wada *et al.*, 1974) was later corrected (Hase *et al.*, 1976*a*).

Biochemical properties

In a study of the comparative immunochemistry of a wide range of bacterial, algal and plant ferredoxins, Tel-Or *et al.* (1977) showed that both ferredoxins reacted equally well with antibody to *Beta vulgaris* ferredoxin, but that ferredoxin I showed greater reactivity with antibody to *Scenedesmus* ferredoxin, whereas ferredoxin II was more reactive with antibody to *S. maxima* ferredoxin. These differences presumably reflect differences in amino acid sequence.

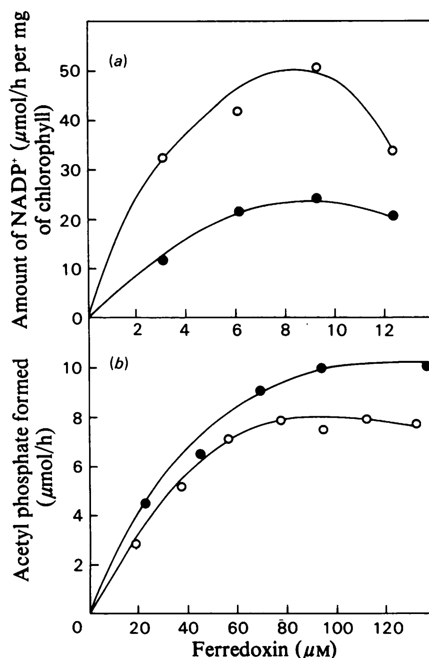

Fig. 1. *Activities of Nostoc strain MAC ferredoxins*
Comparison of ferredoxins I (●) and II (○) as electron carriers (*a*) for $NADP^+$ photoreduction, (*b*) in oxidative decarboxylation of pyruvate. For experimental details see the text.

The biological activities of the ferredoxins in two assay systems were compared. First, activities were assessed by ability of the ferredoxins to catalyse $NADP^+$ photoreduction in barley chloroplasts depleted of native ferredoxin (Fig. 1*a*). The maximum rate supported by ferredoxin II was approx. 50μmol of $NADP^+$ reduced/h per mg of chlorophyll, whereas ferredoxin I was about half as active under identical assay conditions. No $NADP^+$ photoreduction was observed if ferredoxin was omitted, or if saturating concentrations of ferredoxin were present, but the chloroplasts omitted. The decrease in $NADP^+$ photoreduction at high ferredoxin concentration has been noted with *Porphyra umbilicalis* ferredoxin (Andrew *et al.*, 1976) and also occurs, for example, with the ferredoxins from *N. verrucosum* (Shin *et al.*, 1977). Day-to-day reproducibility in assays was checked by inclusion on each occasion of parallel assays using *C. pasteurianum* ferredoxin, which showed intermediate activity compared with the two ferredoxins from *Nostoc* strain MAC, catalysing a maximum rate of 37μmol of $NADP^+$ reduced/h per mg of chlorophyll.

The activities of ferredoxins I and II were also measured by their ability to support pyruvate decarboxylation by the phosphoroclastic system of *C. pasteurianum*, depleted of native ferredoxin (Fig. 1*b*). Some cyanobacteria are known to carry out pyruvate decarboxylation by this system (Leach & Carr, 1971; Bothe & Nolteernsting, 1975). In the *C. pasteurianum* system, ferredoxin I proved to be the more active, though ferredoxin II showed some 75% of its activity. As one might expect, in this assay the native ferredoxin was the most active, though only slightly more so than ferredoxin I.

There are reports of differing activities for the two ferredoxins in *R. rubrum*, where ferredoxin I was three times as effective as ferredoxin II in coupling the reducing power of illuminated chloroplasts to nitrogenase activity (Yoch & Arnon, 1975), and *B. polymyxa*, where ferredoxin II was more effective in an assay of nitrogen fixation (Yoch, 1973). Among the cyanobacteria such comparative studies are still lacking, though the minor ferredoxin of *A. sacrum* proved to have lower activity than the major ferredoxin in supporting $NADP^+$ photoreduction (Hase *et al.*, 1975), whereas ferredoxin II of *N. verrucosum* apparently proved most active in a $NADP^+$-photoreduction assay, and ferredoxin I most active in supporting NADPH–cytochrome *c* reductase activity (Shin *et al.*, 1977).

E.p.r. spectra and redox potentials

The ferredoxins gave the rhombic e.p.r. signal in the reduced state characteristic of two-iron fer-

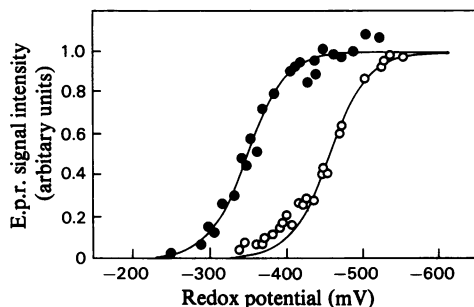

Fig. 2. *Midpoint redox potentials of Nostoc ferredoxins I and II*

Experimental points are the intensity of the e.p.r. signal (g_y) at $g = 1.96$ for reduced *Nostoc* ferredoxins as a function of the redox potential at which the samples, in 0.15M-Tris/HCl, pH8.0, were poised. Data for ferredoxin I (●) and ferredoxin II (○) are superimposed on theoretically derived plots for one-electron-transferring species.

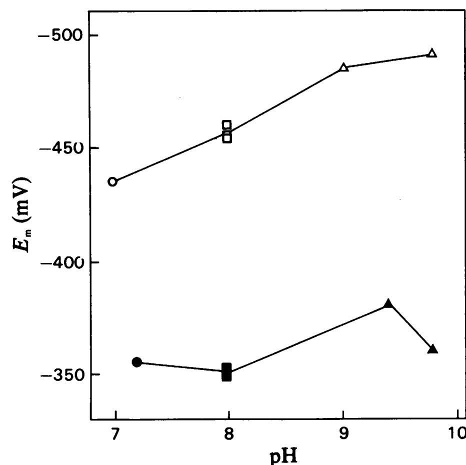

Fig. 3. *Dependence on pH of the midpoint potentials of Nostoc ferredoxins I and II*

Buffers, at a concentration of 0.15M, were: ●, ○, 4-morpholinepropanesulphonic acid/NaOH; ■, □, Tris/HCl; ▲, △, glycine/NaOH. Solid and open symbols are data for ferredoxin I and ferredoxin II respectively.

redoxins from plants or algae. At 22 K the principal g values (±0.005) were measured as $g_x = 1.885$, $g_y = 1.960$, $g_z = 2.053$ for ferredoxin I and $g_x = 1.895$, $g_y = 1.962$, $g_z = 2.056$ for ferredoxin II. The differences were small but reproducible, possibly reflecting different geometry of the iron–sulphur clusters.

The redox behaviour of ferredoxins I and II at pH8.0 as determined by redox titration and e.p.r. spectroscopy is shown in Fig. 2. Clearly there is a difference in midpoint redox potential between the two proteins. Both titrated as one-electron acceptors, with midpoint redox potentials at −350mV and −455mV respectively. Ferredoxin II showed evidence of contamination with a small amount of a species with a midpoint potential of about −400mV. This was probably not due to contaminant ferredoxin I, but to a modified form of ferredoxin II, as it became much more prominent at higher pH values. A comparable difference in redox potentials occurs with the two *S. maxima* ferredoxins (Cammack *et al.*, 1977*b*).

The effect of pH on the midpoint potentials of the two ferredoxins is shown in Fig. 3. Ferredoxin II showed a marked decrease in potential with increasing pH in the range 7.0–9.5, whereas this tendency was not apparent with ferredoxin I. Higher-plant ferredoxins that have been investigated show little variation with pH (Tagawa & Arnon, 1968; Stombaugh *et al.*, 1976). The redox potential of ferredoxin II is close to those of other plant ferredoxins such as spinach (−420mV; Tagawa & Arnon, 1968) at pH 7, but decreases with increasing pH. This is not because the protein undergoes oxidation–reduction by hydrogen transfer, since that would imply that the potential must change by −60mV per pH unit. In no case has this been confirmed for a ferredoxin (Stombaugh *et al.*, 1976), though the Rieske iron–sulphur centre in chromatophores and mitochondria exhibits a pK in the oxidized form at pH8 (Prince & Dutton, 1976) suggested to be caused by hydrogen transfer accompanying electron transfer at higher pH values. *B. polymyxa* ferredoxin II shows a similar change in midpoint potential with pH (Stombaugh *et al.*, 1976) to ferredoxin II from *Nostoc* strain MAC, whereas somewhat smaller changes occur for *B. polymyxa* ferredoxin I and the ferredoxins from *C. pasteurianum* (Stombaugh *et al.*, 1976) and *C. acidi-urici* (Lode *et al.*, 1976*b*). The latter authors suggest that the redox potential of a cluster could be altered by changes in pH or ionic strength of the environment through changes in protein conformation. Another possible explanation is that a group in the vicinity of the iron–sulphur centre in ferredoxin II of *Nostoc* strain MAC with a pK around 8–9 undergoes ionization to produce a negative charge. The presence of this negative charge would make it more difficult to introduce a reducing electron into the centre (cf. Kassner & Yang, 1973), and thereby lower the midpoint potential. A suitable candidate for such a group might be a cysteine residue. If this is so ferredoxin II may contain five cysteine residues, not four as present analyses suggest, the cysteine residue that ionizes being that at position 88 (numbering as in Fig. 4), since cysteine at position 21 is known to be absent.

The existence of two ferredoxins with markedly different redox potentials suggests that one of the

ferredoxins, presumably the predominant ferredoxin I ($E_m = -350$ mV), is adapted to a role additional to that of catalysis of $NADP^+$ photoreduction. It may be noted that *Halobacterium halobium*, which does not carry out photosynthetic electron transport, contains a ferredoxin that shows considerable homology with *N. muscorum* ferredoxin in its amino acid sequence (Hase *et al.*, 1977*c*). In correlating redox potential with biological activity for these ferredoxins we recognize that the assays involve components from other organisms. Thus in the assay of $NADP^+$ photoreduction by barley chloroplasts the native ferredoxin–$NADP^+$ reductase is present. However, it might be noted that similar activity in the comparable spinach chloroplast system was shown by ferredoxins from *S. maxima* (ferredoxin I), spinach, maize and *Scenedesmus obliquus* (Hall *et al.*, 1972). All these ferredoxins have comparable redox potentials (Cammack *et al.*, 1977*b*). It is also known that both the predominant *A. sacrum* ferredoxin and spinach ferredoxin complex equally well with spinach ferredoxin–$NADP^+$ reductase, and catalyse $NADP^+$ photoreduction by spinach chloroplasts equally effectively (Wada *et al.*, 1974).

Ferredoxins can also act as electron donor to nitrite reductase (Ramirez *et al.*, 1965), sulphite

Table 3. *Amino acid sequence of Nostoc ferredoxin I, with the results of analyses of each sample from the sequencer*
Procedures for the analyses are given in Haslett & Boulter (1976). Phenylthiohydantoin derivative of norleucine (50 nmol) was added to each tube of the sequencer fraction collector. This internal standard allowed the quantitative determination of derivatives by g.l.c. The values in parentheses represent yields of residue (in nmol) from 500 nmol of carboxymethylated protein.

Sequence position	Chromatographic identification: T.l.c.	Chromatographic identification: G.l.c.	Identification of parent amino acid after regeneration and dansylation	Residue
1	Ala	Ala (274.0)		Alanine
2	Thr	Gly (105.0)	α-Aminobutyrate*, Ala, Gly	Threonine
3	Val	Val (106.0)		Valine
4	Tyr		Tyr*, Ala, Gly	Tyrosine
5	Lys		Lys*, Ala, Gly	Lysine
6	Val*, Ala	Val (86.0), Ala (15.3)		Valine
7	Thr		α-Aminobutyrate*, Pro, Val, Ala, Gly	Threonine
8	Leu*, Ala	Leu (71.0)		Leucine
9	Val*, Leu	Val (84.0), Leu (10.3)		Valine
10	Asp	Asp (24.0)		Aspartic acid
11	Gln*, Glu, Asp		Glu*, Gly, Asp, Ala	Glutamine
12	Glu	Glu (18.5)		Glutamic acid
13	Gly*, Glu	Gly (19.3)		Glycine
14	Thr		α-Aminobutyrate*, Gly	Threonine
15	Glu	Glu (6.0)		Glutamic acid
16	Thr		α-Aminobutyrate*, Glu, Gly, Ala	Threonine
17	Thr		α-Aminobutyrate*, Gly, Ala	Threonine
18	Leu/Ile*, Thr	Ile (25.0)		Isoleucine
19	Asp	Asp (14.6)		Aspartic acid
20	Val*, Asp	Val (26.9)		Valine
21	Pro*, Val, Asp	Pro (11.0), Val (14.0)	Pro*, Ala, Gly, Val*	Proline
22	Asp	Asp (18.4)		Aspartic acid
23	Asp	Asp (37.0)		Aspartic acid
24	Glu*, Asp	Glu (13.6), Asp (6.7)		Glutamic acid
25	Tyr*, Asp, Glu		Tyr*, Pro, Asp	Tyrosine
26	Leu/Ile	Ile (15.0)		Isoleucine
27	Leu/Ile	Leu (10.0)		Leucine
28	Asp	Asp (7.6)		Aspartic acid
29	Leu/Ile	Ile (14.0)		Isoleucine
30	Ala*, Asp	Ala (8.0)		Alanine
31	Glu*, Ala	Glu (7.1)		Glutamic acid
32	Asp		Asp*, Glu, Gly, Ala	Aspartic acid
33	Gln*, Glu		Glu*, Gly, Ala	Glutamine
34	Gly*, Glu		Gly*, Asp, Ala	Glycine
35	Leu/Ile	Leu (4.0)		Leucine
36	Asp		Asp*, Gly, Ala	Aspartic acid
37	Leu/Ile	Leu (2.2)		Leucine

* Residue in greater concentration as estimated qualitatively

reductase (Asada *et al.*, 1971) and glutamate synthase (Lea & Miflin, 1974) in higher plants, and also in the phosphoroclastic reaction (Leach & Carr, 1971) and as electron donor to nitrogenase (Smith *et al.*, 1971) in cyanobacteria. Other than $NADP^+$ photoreduction it is not known whether any of these systems operate in *Nostoc* strain MAC. In view of its genetic position *Nostoc* strain MAC should be able to fix nitrogen, but apart from the original report (Bowyer & Skerman, 1968) this has not been demonstrated. Apart from *Nostoc* strain MAC, only for *B. polymyxa* (Stombaugh *et al.*, 1973) and *A. vinelandii* (Yoch & Arnon, 1972) have both the redox potentials and biological activities of the two ferredoxins that occur been studied. Ferredoxins I and II differ in redox potential by 40mV, but show similar activity in a nitrogenase assay, $NADP^+$ photoreduction, and the phosphoroclastic reaction (Yoch & Arnon, 1972; Yoch, 1973; Stombaugh *et al.*, 1973), apart from *B. polymyxa*, where ferredoxin II proved more active in the first of these activities (Yoch, 1973). The three oligomeric forms of the ferredoxin in *Desulphovibrio gigas* have redox potentials between −430mV and −455mV for transitions between the oxidation states C^{2-} and C^{3-} (see Cammack *et al.*, 1977*a*) and at saturating concentrations showed similar activity in catalysing reduction of sulphite by molecular hydrogen, though at low concentration ferredoxin II was the most active (Bruschi *et al.*, 1976).

Amino acid sequence

The *N*-terminal sequence of both *Nostoc* strain MAC ferredoxins, isolated from photoautotrophically grown cells, were determined on a sequencer

Table 4. *Amino acid sequences of Nostoc ferredoxin II with the results of analyses of each sample from the sequencer*
Procedures for the analyses are given in Haslett & Boulter (1976). Phenylthiohydantoin derivative of norleucine (50nmol) was added to each tube of the sequencer fraction collector. This internal standard allowed the quantitative determination of derivatives by g.l.c. The values in parentheses represent yields of residue (in nmol) from 500nmol of carboxymethylated protein.

Sequence position	Chromatographic identification		Identification of parent amino acid after regeneration and dansylation	Residue
	T.l.c.	G.l.c.		
1	Ala	Ala (136.0)		Alanine
2	Thr		α-Aminobutyrate*, Gly, Val	Threonine
3	Tyr		Tyr*, Ala, Gly	Tyrosine
4	Lys		Lys*, Ala, Gly, Tyr	Lysine
5	Val	Val (79.0)		Valine
6	Asp		Asp*, Ala, Val	Aspartic acid
7	Leu	Leu (61.0)		Leucine
8	Phe	Phe (42.0)		Phenylalanine
9	Asn*, Asp		Asp*, Gly, Ala, Tyr, Phe	Asparagine
10	Ala	Ala (58.0)		Alanine
11	Ala	Ala (69.0)		Alanine
12	Glu	Glu (49.2)		Glutamic acid
13	Gly*, Glu	Gly (21.8)		Glycine
14	Leu	Leu (36.0)		Leucine
15	Asp	Asp (32.0)		Aspartic acid
16	Glu	Glu (29.4)		Glutamic acid
17	Thr		α-Aminobutyrate*, Ala	Threonine
18	Ile	Ile (47.5)		Isoleucine
19	Glu	Glu (21.7)		Glutamic acid
20	Val*, Glu	Val (37.0)		Valine
21	Pro	Pro (20.2)		Proline
22	Asp*, Glu	Asp (13.0)		Aspartic acid
23	Asp*, Ala	Asp (10.8)		Aspartic acid
24	Glu		Glu*, Asp, Gly, Ala	Glutamic acid
25	Tyr*, Glu		Tyr*, Ala, Gly, Glu, Asp	Tyrosine
26	Ile*, Tyr	Ile (15.4)		Isoleucine
27	Leu/Ile*, Glu	Leu (10.6)	Leu*, Ala, Gly, Asp	Leucine
28	Asp*	Asp (4.1)		Aspartic acid
29	Ala*, Asp, Glu	Ala (6.6)		Alanine
30	Ala*, Asp, Glu	Ala (10.2)		Alanine
31	Glu		Glu*, Ala	Glutamic acid
32	(Glu)		Glu	(Glutamic acid)

* Residue in greater concentration as estimated qualitatively.

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                              1             5                10                15
Nostoc strain MAC, I     Ala-Thr-Val-Tyr-Lys-Val-Thr-Leu-Val-Asp-Gln———Glu-Gly-Thr-Glu-Thr-Thr-Ile-
Nostoc strain MAC, II    Ala-Thr———Tyr-Lys-Val-Asp-Leu-Phe-Asn-Ala-Ala-Glu-Gly-Leu-Asp-Glu-Thr-Ile-
N. muscorum              Ala-Thr———Phe-Lys-Val-Thr-Leu-Ile-Asn-Glu-Ala-Glu-Gly-Thr-Lys-His-Glu-Ile-
S. maxima                Ala-Thr———Tyr-Lys-Val-Thr-Leu-Ile-Ser-Glu-Ala-Glu-Gly-Ile-Asn-Glu-Thr-Ile-
A. sacrum                Ala-Ser———Tyr-Lys-Val-Thr-Leu-Lys-Thr-Pro———Asp-Gly———Asp-Asn-Val-Ile-

                           20                25                30                35
Nostoc strain MAC, I      Asp-Val-Pro-Asp-Asp-Glu-Tyr-Ile-Leu-Asp-Ile-Ala-Glu-Asp-Gln-Gly-Leu-Asp-Leu
Nostoc strain MAC, II     Glu-Val-Pro-Asp-Asp-Glu-Tyr-Ile-Leu-Asp-Ala-Ala-Glu(Glu)
N. muscorum               Glu-Val-Pro-Asp-Asp-Glu-Tyr-Ile-Leu-Asp-Ala-Ala-Glu-Glu-Glu-Gly-Tyr-Asp-Leu
S. maxima                 Asp-Cys-Asp-Asp-Asp-Thr-Tyr-Ile-Leu-Asp-Ala-Ala-Glu-Glu-Ala-Gly-Leu-Asp-Leu
A. sacrum                 Thr-Val-Pro-Asp-Asp-Glu-Tyr-Ile-Leu-Asp-Val-Ala-Glu-Glu-Glu-Gly-Leu-Asp-Leu
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Fig. 4. *Comparison of the amino acid sequences of cyanobacterial ferredoxins*

Comparison of the amino acid sequences of *Nostoc* strain MAC ferredoxins I and II is made with those of the ferredoxins from *N. muscorum* (Hase *et al.*, 1976*b*), *S. maxima* (Tanaka *et al.*, 1975) and *A. sacrum* (Wada *et al.*, 1974). The sequence for *S. platensis* (Wada *et al.*, 1975*b*) differs from *S. maxima* in this segment only in that residue 10 is aspartic acid.

by using 500nmol of carboxymethylated protein (Tables 3 and 4). A similar analysis of ferredoxin I from heterotrophically grown cells yielded an *N*-terminal sequence identical with that of ferredoxin I from cells grown photoautotrophically, confirming that the same ferredoxin I is produced under both growth conditions. Unambiguous identification of all residues was possible, although the evidence for residue 32 of ferredoxin II is less firm. After 32 residues (ferredoxin II) and 37 residues (ferredoxin I) the build-up of background prevented further unequivocal identifications from being made. In the first 32 residues there are 12 amino acid differences between ferredoxins I and II. The partial sequences are compared (Fig. 4) with the corresponding sequences for the other cyanobacterial ferredoxins so far reported. With the other cyanobacteria only the predominant ferredoxin has so far been sequenced. For the residues considered, the sequences for *S. maxima* and *Spirulina platensis* are identical except for residue 10, which in *S. platensis* ferredoxin is aspartic acid. Hence the differences between *Nostoc* strain MAC ferredoxins I and II are very considerable by comparison. The sequencer did not resolve residues 37–50 in the ferredoxins, a segment that almost certainly would have included three of the four invariant cysteine residues involved in binding the iron–sulphur centre. For the segment shown there are 10–12 amino acid differences between *Nostoc* strain MAC ferredoxin I and the other cyanobacterial ferredoxins. A similar trend is seen for ferredoxin II. The ferredoxin from *N. muscorum* shows only slightly greater resemblance to the ferredoxins from *Nostoc* strain MAC than to the other cyanobacterial ferredoxins. Evolutionary relationships of some of these organisms, as assessed from ferredoxin sequences, are discussed elsewhere (Wada *et al.*, 1975*a*; Hase *et al.*, 1976*a*,*b*; but cf. Fitch & Yasunobu, 1975).

Among points of interest in these sequences is that cysteine, commonly found at position 21 in plant-type ferredoxins, is replaced in both *Nostoc* strain MAC ferredoxins by valine. This difference also occurs in *N. muscorum* (Hase *et al.*, 1976*b*) and *A. sacrum* (Hase *et al.*, 1976*a*) ferredoxins, confirming a suggestion from the sequence of *Equisetum* ferredoxin (Aggarwal *et al.*, 1971; Kagamiyama *et al.*, 1975) that cysteine at this position is not involved in binding the chromophore. Both *Nostoc* strain MAC ferredoxins contain two extra amino acid residues compared with higher-plant ferredoxins. In ferredoxin II these are alanine and leucine at positions 12 and 15, whereas in ferredoxin I valine occurs at position 3 and threonine at position 15. Two additional residues in this segment at positions 12 and 15 also occur in the ferredoxins from *N. muscorum*, *S. maxima* and *Porphyra umbilicalis* (Andrew *et al.*, 1976), though the particular amino acids inserted vary somewhat. The addition of two extra residues relative to other ferredoxins so far sequenced correlates in all cases with absence of proline from position 11. Possibly when proline is absent two additional residues are necessary to preserve the functional integrity or stability of the holoprotein. The ferredoxins from *Nostoc* strain MAC, like that of *S. maxima* (Hall *et al.*, 1972), are very stable proteins.

The different redox potentials of the two *Nostoc* strain MAC ferredoxins presumably result from the different conformations of the molecules induced by changes in amino acid sequence, these influencing the geometry and electronic properties of the iron–sulphur cluster, which probably lies near the surface of the molecule (Ogawa *et al.*, 1977). Thus substitution of histidine or tryptophan at position 2 in *C. acidi-urici* ferredoxin in place of tyrosine causes a change in redox potential, and of stability, of the ferredoxin (Lode *et al.*, 1976*a*,*b*), and, for the latter substitution, lower activity in assays of the phosphoroclastic system and cytochrome *c* reduction (Lode *et al.*, 1976*a*).

At the present time the full sequences of four cyanobacterial ferredoxins are known; those from *S. maxima* (ferredoxin I; Tanaka *et al.*, 1975), *S. platensis* (Wada *et al.*, 1975*b*), *A. sacrum* (major ferredoxin; Hase *et al.*, 1976*a*) and *N. muscorum* (major ferredoxin; Hase *et al.*, 1976*b*). Comparable studies on the two *Nostoc* strain MAC ferredoxins would evidently be of value in elucidating structure–activity relationships in these interesting ferredoxins.

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