Properties and structure of the soluble ferredoxin from *Synechococcus* 6301 (*Anacystis nidulans*)

Relationship to gene sequences

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Photoautotrophic cultures of the unicellular cyanobacterium *Synechococcus* 6301 (*Anacystis nidulans*) possessed a single [2Fe-2S] ferredoxin with a midpoint redox potential of -385 mV. Determination of the amino acid sequence of the ferredoxin showed that it consisted of 98 residues, with methionine and tryptophan both absent, and with only the four cysteine residues that are required to co-ordinate the iron-sulphur cluster. Comparisons with other ferredoxin sequences showed that most resemblance was to those from filamentous cyanobacteria, with up to 87% homology. There was less resemblance to the ferredoxins of unicellular cyanobacteria, with 25 differences when compared with that from another *Synechococcus* sp. However, the sequence of *Synechococcus* 6301 ferredoxin was identical with that derived for a gene sequence for a putative ferredoxin from the genotypically closely related *Synechococcus* 7942 (*Anacystis nidulans* R2). In contrast, the sequence showed substantial differences from that corresponding to a putative ferredoxin gene from *Synechococcus* 6301 reported by Cozens & Walker [(1988) *Biochem. J.* 252, 563–569].

INTRODUCTION

Ferredoxins from more than 20 cyanobacteria have been isolated and characterized to various extents (see Rogers, 1987); 14 have been sequenced, and all show considerable homology, particularly at the sequences around the four cysteine residues that co-ordinate the iron-sulphur cluster.

The majority of cyanobacteria appear to possess a single soluble ferredoxin. However, several cyanobacteria possess two ferredoxins; this is the case for *Aphanothece sacrum* (Hase *et al.*, 1975), *Nostoc* strain MAC (Hutson & Rogers, 1975), *Nostoc muscorum* (Hase *et al.*, 1976), *Nostoc verrucosum* (Shin *et al.*, 1977), *Spirulina maxima* (Cammack *et al.*, 1977) and most recently *Anabaena variabilis* (Schrautemeier & Bohme, 1985), *Microcystis aeruginosa* (Cohn *et al.*, 1985) and *Aphanizomenon flos-aquae* (Cohn *et al.*, 1985; Matsubara & Wada, 1988). This group includes examples of both unicellular and filamentous cyanobacteria, and within the latter group heterocystous and non-heterocystous types. The two proteins are genetically independent isoenzymes (Hase *et al.*, 1978) and probably arise as a result of duplication of the primitive ferredoxin gene with divergent evolution leading to the development of proteins with distinctive structural and possibly functional properties (Takaichi *et al.*, 1978).

When two ferredoxins are present their relative amounts differ widely from approaching equal amounts in *Nostoc verrucosum* (Shin *et al.*, 1977), 2:1 for *Nostoc* strain MAC (Hutson *et al.*, 1978) and 5:1 for *Aphanothece sacrum* (Hase *et al.*, 1975). In the others the second

ferredoxin was only present in small quantities, posing the question of contamination by a subspecies where the organism has been isolated from natural sources.

The possibility that in heterocystous cyanobacteria the minor ferredoxin is located in the heterocysts has been suggested (Schrautemeier & Bohme, 1985). In the case of *Aphanizomenon flos-aquae* the minor ferredoxin may be membrane-bound (Cohn *et al.*, 1985; Matsubara & Wada, 1988).

Synechococcus 6301 (formerly *Anacystis nidulans*) is a unicellular cyanobacterium of popular choice for biochemical investigations. Its ferredoxin was one of the first to be isolated from an oxygenic photosynthetic bacterium (Yamanaka *et al.*, 1969), though its properties have never been fully characterized. We now report the amino acid sequence of *Synechococcus* 6301 ferredoxin. While this work was in progress the nucleotide sequence of a ferredoxin gene from the cyanobacterium was being investigated independently (Cozens & Walker, 1987, 1988), and similar data for a ferredoxin gene from the closely related *Synechococcus* 7942 were the subject of two reports (van der Plas *et al.*, 1986b; Reith *et al.*, 1986). The possible relationships of these sequences are discussed.

METHODS

Organism

Anacystis nidulans was obtained by Dr. A. J. Smith in this laboratory from Dr. M. M. Allen and is the organism deposited subsequently in the Paris Culture Collection as *Synechococcus* P.C.C. 6301. A culture was also obtained

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from Professor A. E. Walsby, Department of Botany, University of Bristol, Bristol, U.K.

Isolation of ferredoxin

In the largest preparation the soluble ferredoxin present was isolated from a total of 160 litres of a photoautotrophically grown culture (Smith *et al.*, 1967). The yield was some 280 g of cell paste, which gave 60 g of acetone-dried powder. This was homogenized in 0.15 M-Tris/HCl buffer, pH 7.4, and polynucleotides were degraded by deoxyribonuclease and ribonuclease treatment. The ferredoxin was isolated by successive $(\text{NH}_4)_2\text{SO}_4$ fractionation, anion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-50. The yield was 150 mg of ferredoxin, measured by weighing after dialysis against distilled water and freeze-drying. The methods are those in routine use in our laboratory at University College of Wales, Aberystwyth, and have been described previously in detail (see, e.g., Hutson *et al.*, 1978).

Attempts to isolate a membrane-bound ferredoxin followed the methods used by Cohn *et al.* (1985) with another cyanobacterium, *Aphanizomenon flos-aquae*.

Homogeneity of the ferredoxin was confirmed by polyacrylamide-gel electrophoresis; gels in the range 10–25% (w/v) acrylamide containing 3.6% of total acrylamide as *NN'*-methylenebisacrylamide were used (Hutson *et al.*, 1978).

Midpoint redox potential

Midpoint redox potential was determined in collaboration with Dr. R. Cammack (Department of Plant Sciences, King's College, University of London, London, U.K.) by potentiometric titration by monitoring the increase in the e.p.r. signal (g_y) at $g = 1.96$ as the ferredoxin was progressively reduced by addition of $\text{Na}_2\text{S}_2\text{O}_4$ under strictly anaerobic conditions to adjust potentials in the presence of suitable mediators and a platinum electrode. The methodology has been described previously (Cammack *et al.*, 1977).

Amino acid sequence

The amino acid sequence of the ferredoxin was determined by *N*-terminal manual Edman degradation of the carboxymethylated ferredoxin, *C*-terminal analysis with carboxypeptidase Y, and manual Edman degradation of peptides derived by either tryptic or staphylococcal-proteinase digestion. Sequencing of the peptides obtained by supplementary chymotryptic digestion of some of the larger tryptic and proteinase peptides also contributed to the determination. The experimental protocols have been described previously on a number of occasions (see, e.g., Hase *et al.*, 1982).

RESULTS AND DISCUSSION

Isolation and properties

During chromatography on the DEAE-cellulose column a single reddish-brown ferredoxin was observed and the purified ferredoxin gave a single band on analytical polyacrylamide-gel electrophoresis. The absorption spectrum was typical of a $[\text{Fe-2S}]$ ferredoxin with maxima at 276, 282 (shoulder), 331, 423 and 464 nm. The A_{331}/A_{276} ratio for a ferredoxin is dependent on the aromatic amino acid content, and the chromo-

phore also contributes to the u.v. absorption. The value of 0.75 for this ratio for *Synechococcus* 6301 ferredoxin is among the highest for a cyanobacterial ferredoxin. The molar absorption coefficient at 432 nm based on a solution of a known weight of ferredoxin was $8500 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This value was significantly higher than the value of $6000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ reported for the initial isolation of the ferredoxin (Yamanaka *et al.*, 1969). The activity of the ferredoxin as mediator in biological assays has been reported previously (Fitzgerald *et al.*, 1980).

The data from a potentiometric titration gave a good fit to the theoretically derived plot for a one-electron-transferring species except for some departure at lowest potentials. This could be due to contamination with a small amount of a species with a midpoint potential of about -300 mV , but might also be explained by a modified form of the ferredoxin or a salt effect. The midpoint potential of -385 mV at pH 7.0 for the ferredoxin is similar to that for the ferredoxins from a number of other cyanobacteria, including *Synechococcus* 6307 (Rogers, 1987), and it shows similar activity in biological assays (Fitzgerald *et al.*, 1980).

Amino acid sequence

The amino acid sequence of the ferredoxin was derived as shown in Fig. 1 from initial manual Edman degradation and carboxypeptidase digestion of the carboxymethylated ferredoxin, and subsequent Edman degradation of tryptic and staphylococcal-proteinase peptides and of chymotryptic peptides derived from these. The only problem was lack of an overlap in the sequence of tryptic peptide T2 adjoining peptide T3 at residues 52–53. However, the sequences could be aligned with certainty on the basis of homology with the other cyanobacterial ferredoxins that have been sequenced (see Rogers, 1987) and in particular the invariant cysteine residues at positions 46 and 49 in peptide T2. Moreover the sequence was entirely compatible with amino acid composition analyses of the peptides. The close agreement of the amino acid composition derived from the sequence with that reported originally (Yamanaka *et al.*, 1969), except for uncertainty as to the number of cysteine residues in the latter analysis, indicated that these ferredoxins corresponded.

The ferredoxin was 98 residues long, and possessed only the four cysteine residues that are needed to co-ordinate the iron-sulphur cluster; no methionine or tryptophan was present.

The sequence of *Synechococcus* 6301 ferredoxin shows most resemblance, not to the ferredoxins of other unicellular cyanobacteria, as might be expected, but to those from filamentous species. The closest identity is to the ferredoxins from the filamentous heterocystous species *Aphanizomenon flos-aquae*, *Chlorogloeopsis fritschii* and *Mastigocladus laminosus* (see Rogers, 1987), and to the sequence derived from a ferredoxin gene identified in *Anabaena variabilis* A.T.C.C. 29413 (van der Plas *et al.*, 1986a). For these, there are only 13–15 differences from *Synechococcus* 6301 ferredoxin comparing amino acids at each position in the sequences aligned for maximum homology. There are 25 differences even comparing *Synechococcus* 6301 ferredoxin with that from another *Synechococcus* sp. (Hase *et al.*, 1983).

While the present work was in progress the nucleotide sequence of a ferredoxin gene from *Synechococcus* P.C.C. 7942 (*Anacystis nidulans* R2) was the subject of

Fig. 1. Summary of the sequence determination of *Synechococcus* 6301 ferredoxin

T and S refer to tryptic peptides and peptides derived by staphylococcal proteinase respectively, and C refers to chymotryptic peptides derived from these. The amino acid sequence was determined by a combination of manual Edman degradation of the carboxymethylated ferredoxin (→ above residues) or of derived peptides (→ below residues; ---→ ambiguous identification), and carboxypeptidase digestion (↖). Cys (☆) was determined as carboxymethylcysteine.

two reports. *Anacystis nidulans* R2 is a transformable strain exploited in gene manipulation procedures. In one case, the gene was isolated from a cosmid library by using the ferredoxin gene of *Anabaena variabilis* A.T.C.C. 29413 as a probe (van der Plas *et al.*, 1986b). In the other (Reith *et al.*, 1986) the gene was obtained by using oligonucleotide probes corresponding to conserved regions of *Synechocystis* 6714 ferredoxin. Apart from the *N*-terminal methionine residue, which presumably will be subject to post-translational cleavage, the sequence is identical with that of *Synechococcus* 6301 ferredoxin. Studies of genetic relationships (Wilmotte & Stam, 1984) have supported the close resemblance of these two

Synechococcus strains, which are genotypically almost identical with each other and with *Synechococcus* P.C.C. 7943 (*Anacystis nidulans* 602). This observation is now underlined at the biochemical level in the identical sequences of their ferredoxins.

Recent papers (Cozens & Walker, 1987, 1988) report the identification and structural elucidation of a ferredoxin gene in *Synechococcus* 6301, identified as such from the sequence homology about the invariant cysteine residues. Even cursory examination shows that the amino acid sequence derived from the nucleotide sequence is markedly different from the sequence for the isolated ferredoxin. The differences between ferre-

Fig. 2. Plot of hydropathy distribution for the ferredoxin isolated from *Synechococcus* 6301 (—) and the putative ferredoxin gene product (---)

Plots were based on Kyte & Doolittle indices and an amino acid window of 11 units.

doxin sequences, based on amino acid or nucleotide sequences as appropriate, for the *Synechococcus* species that have been investigated are shown in Table 1. The ferredoxin corresponding to the *Synechococcus* 6301 gene is diverse from all the cyanobacterial ferredoxins sequenced so far, with 47–61 differences (neglecting an eight-residue C-terminal extension) depending on the particular ferredoxin being compared, with most similarity to *Chlorogloeopsis fritschii* ferredoxin. Closest resemblance in a mutation data matrix analysis using a database was to the ferredoxin from *Synechococcus* sp. (given as *Synechococcus lividus* in the database of the Protein Information Resource, National Biomedical Research Foundation, Washington, DC, U.S.A.; but see Hase *et al.*, 1983). However, the differences are still greater than between any pair of cyanobacterial ferredoxin sequences, even excluding consideration of the

Table 1. Amino acid difference matrix of *Synechococcus* ferredoxins

In the nucleotide sequences of the genes the N-terminal methionine and any extension at the C-terminus have been omitted in the comparisons. The sequences were arranged for maximum homology, and gaps introduced for this purpose were each counted as 1.

		(a)	(b)	(c)	(d)
<i>Synechococcus</i> 6301 ferredoxin	(a)				
<i>Synechococcus</i> 6301 ferredoxin gene	(b)	54			
<i>Synechococcus</i> 7942 ferredoxin gene	(c)	0	54		
<i>Synechococcus</i> sp. ferredoxin	(d)	25	52	25	

extension at the C-terminus. Although this latter feature is also seen in the *Halobacterium* sp. ferredoxins, these are also diverse in sequence comparisons, with only about 20% homology. For comparison, the differences between *Synechococcus* 6301 ferredoxin and other cyanobacterial ferredoxins range from 13 to 28.

A significant difference between the isolated *Synechococcus* 6301 ferredoxin and the ferredoxin gene sequence is seen when hydropathy plots are considered (Fig. 2). That for the ferredoxin is very similar to the hydropathy plots for other cyanobacterial proteins, including ferredoxins I and II from *Nostoc* strain MAC and from *Aphanothece sacrum*, except that the hydrophobic sequence at residues 70–80 is somewhat more pronounced. In contrast, the ferredoxin corresponding to the nucleotide sequence has a distinctive pattern of amino acid distribution; the hydrophobic sequence at residues 70–80 is less pronounced and in particular three of the invariant cysteine residues (positions 39, 41 and 46) are in a significantly more hydrophobic environment.

Comparison of the amino acid sequence of the ferredoxin corresponding to the gene with the X-ray-crystallographic data for *Spirulina platensis* ferredoxin suggests that the major structural features (the β -sheet component, the active-site region and the α -helix at residues 26–33) will be present. However, the distribution of positive and negative charges on the surface of the molecule would be distinct from other cyanobacterial ferredoxins, possibly suggesting a difference in function.

The histories of the *Synechococcus* strain in the present study and that used by Cozens & Walker (1987) suggest they both correspond to that deposited in the Pasteur Culture Collection as strain P.C.C. 6301. In the strain held at Cambridge a second ferredoxin gene that would hybridize with the ferredoxin gene identified was not located (J. E. Walker, personal communication). However, this could be because the ferredoxins are only distantly related, and this is supported by the difference in the nucleotide sequence that would correspond to the isolated ferredoxin compared with the gene sequence. The converse problem is the apparent absence of a second ferredoxin in cell extracts of the *Synechococcus* 6301 held at University College of Wales, Aberystwyth, and that obtained from the University of Bristol Collection (which corresponds to that used by the Cambridge group), though there is evidence from mRNA transcription studies (J. E. Walker, personal communication) that the appropriate gene is expressed. The second ferredoxin would be less acidic, possessing 14 residues of glutamic acid plus aspartic acid compared with 18 residues in this category for the isolated ferredoxin. The proteins should therefore separate readily on DEAE-cellulose chromatography. If a second soluble ferredoxin was present its quantities must be extremely small, since it could not be detected during developing of a DEAE-cellulose column loaded with more than 150 mg of crude ferredoxin after the initial $(\text{NH}_4)_2\text{SO}_4$ fractionation during purification. However, in some cyanobacteria apparently containing two ferredoxins the minor ferredoxin was present in only trace amounts; this was the case with *Spirulina maxima* (Cammack *et al.*, 1977), *Microcystis aeruginosa* (Cohn *et al.*, 1985) and *Aphanizomenon flos-aquae* (Cohn *et al.*, 1985; Matsubara & Wada, 1988). All were cyanobacteria harvested from blooms occurring naturally. In such blooms of *Microcystis aeruginosa* the ratio of the two ferredoxins, when

a second was detectable, was apparently dependent on environmental factors (Cohn *et al.*, 1985). Thus expression of ferredoxin genes may be controlled in response to environmental conditions and the genes may be independently regulated (Hutber *et al.*, 1977). This may explain our inability to detect a ferredoxin corresponding to the gene sequence. However, there is an alternative explanation. In *Aphanizomenon flos-aquae* the major soluble ferredoxin I in natural blooms of the cyanobacterium was accompanied by trace amounts of a second ferredoxin. Treatment of a photosynthetic membrane preparation to release membrane-bound components released significantly higher quantities of ferredoxin II, which, other than being membrane-associated, appeared to be a typical [2Fe-2S] ferredoxin (Cohn *et al.*, 1985; Matsubara & Wada, 1988). In membrane-associated proteins the recognition in hydrophathy plots of their sequences of a hydrophobic region of some 20 residues suggests these portions of the peptide chain span the membrane. The hydrophathy plot of the ferredoxin sequence derived from the *Synechococcus* 6301 gene (Fig. 2) suggests that the iron-sulphur cluster could be membrane-located. The distribution of the four cysteine residues present in the sequence suggests this would be a [2Fe-2S] cluster; membrane-bound iron-sulphur centres in photosynthetic lamellae include the Rieske iron-sulphur protein [2Fe-2S] centre, which is located in the intermediate electron-transport chain between photosystem I and photosystem II (see Evans, 1982).

The Rieske iron-sulphur protein of the cytochrome *b₆f* complex of spinach thylakoids has been isolated and partly sequenced (Pfefferkorn & Meyer, 1986). This is a 20 kDa protein, about twice the size of the putative *Synechococcus* gene product. The sequence around the iron-binding centre shows no homology to that of the gene product, but does to the corresponding sequences about the cysteine residues of the iron-sulphur centre of the cytochrome *bc₁* complexes from the fungus *Neurospora crassa* (Harnisch *et al.*, 1985) and the photosynthetic bacterium *Rhodospseudomonas spheroides* (Gabbellini & Sebald, 1986).

One of the three iron-sulphur centres associated with photosystem I may be a [2Fe-2S] cluster, but is probably associated with a large subunit (59–63 kDa) in photosystem I particles (Warden & Golbeck, 1986).

Our attempts to isolate a membrane-bound iron-sulphur centre from *Synechococcus* 6301 by using methods found to be effective for cyanobacterial cytochrome *f* isolation (Ho & Krogmann, 1980) and that apparently released a second ferredoxin from *Aphanizomenon flos-aquae* (Cohn *et al.*, 1985) have so far been unsuccessful. This does not discount the possibility that the putative ferredoxin gene product is a membrane-bound protein. For example, the iron-sulphur cluster and characteristic absorption spectrum may be lost during the isolation procedure, making the protein difficult to recognize.

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