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PROPERTIES AND AMINO ACID SEQUENCE OF THE FERREDOXIN FROM THE UNICELLULAR CYANOBACTERIUM *SYNECHOCOCCUS* 6307

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Key Word Index—*Synechococcus* 6307; Cyanophyceae; ferredoxin; redox potential; amino acid sequence; taxonomy.

Abstract—A ferredoxin was purified from the unicellular cyanobacterium *Synechococcus* PCC 6307, notable for its unusually high mol% GC. The sequence of 98 amino acids was resolved from investigations of lysylendopeptidase peptides with supplementary data from chymotryptic peptides and carboxypeptidase treatment in some cases. A comparison with the sequences of 18 other cyanobacterial ferredoxins, including some structures derived from gene sequencing is presented. This comparison suggests that on the basis of specific features in the sequence and a phylogenetic tree, *Synechococcus* 6307 more closely resembles the filamentous cyanobacteria rather than unicellular cyanobacteria of other genera.

INTRODUCTION

Ferredoxins are small electron-transfer proteins with a negative redox potential. In oxygenic photosynthetic organisms they have a crucial role in photosynthetic electron flow but also function directly or indirectly in a range of other cell systems; in cyanobacteria, ferredoxin-dependent reactions include nitrogenase, hydrogenase, glutamate synthase, pyruvate-ferredoxin oxidoreductase and thioredoxin-ferredoxin reductase [1]. Cyanobacteria examined have invariably contained one, or in some cases two, ferredoxins which possess a single [2Fe-2S] cluster; however, membrane-bound iron-sulphur centres also occur and may include [4Fe-4S] clusters [2].

Ferredoxins from a considerable number of cyanobacteria have been isolated and characterised to varying extent; 14 have been sequenced [see 1,3] and the sequences of three others have been deduced from gene sequences [4–7] with a fourth [8] possibly being that of a membrane-bound iron-sulphur centre rather a soluble ferredoxin [9]. The broad division into unicellular and filamentous cyanobacteria is generally supported by specific features in the sequences and in phylogenetic trees [10]. However, the sequences of the ferredoxin from the unicellular cyanobacterium *Synechococcus* 6301 [11] proved anomalous in showing closest identity to the ferredoxins from the filamentous heterocystous species *Aphanizomenon flos-aque*, *Chlorogloeopsis fritschii* and *Mastigocladus laminosus*. We now report the sequence of the ferredoxin from the related cyanobacterium *Synechococcus* 6307, notable in this group of prokaryotes for possessing the highest mol% GC of 71% in its DNA base composition [12], and discuss evolutionary and taxonomic relationships based on cyanobacterial ferredoxin structures.

RESULTS AND DISCUSSION

Isolation and properties

Some 240 g of the cell paste accumulated from bulk cultures totalling 128 l of *Synechococcus* 6307 gave 47 g of an acetone-dried powder. During isolation of the ferredoxin from this only a single ferredoxin was evident during column chromatography on DEAE-cellulose; the final yield of ferredoxin was *ca* 100 mg. The absorption spectrum was characteristic of a [2Fe-2S] ferredoxin with maxima at 276, sh 282, 329, 425 and 460 nm, the latter an extended plateau rather than a distinct maximum. The A_{max}/A_{276} nm ratios for the maxima were 0.78, 0.60 and 0.55, respectively. The ratios would be worsened dramatically by the presence of other proteins and the high A_{329}/A_{276}nm ratio therefore suggested the ferredoxin was homogeneous, a conclusion confirmed by analytical polyacrylamide gel electrophoresis which at a range of concentrations showed only one Coomassie Blue stained band.

The data from a potentiometric titration gave a very good fit to the theoretically derived plot for a one-electron-transferring species, confirming the absence of any other absorbing species of significantly different E_m. The plot of the data as log ox/red vs E_h (Fig. 1) shows a value for E_m of -395 mV and a slope of 55 mV, which was close to the theoretical value of 59 mV for a one-electron transfer given by the Nernst equation. The E_m is at the low end of the range for cyanobacterial ferredoxins,

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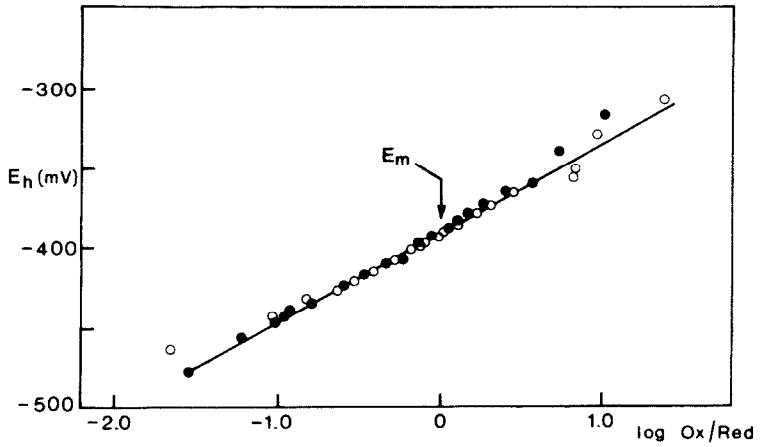

Fig. 1. Midpoint redox potential of *Synechococcus* 6307 ferredoxin. The plot is log ox/red versus cell potential for a reductive titration (○) and a successive oxidative titration (●) at pH 8.0.

and was somewhat lower than the E_m of −380 mV obtained for *Synechococcus* 6301 ferredoxin [9].

Sequence determination

The amino acid sequence summarized in Fig. 2 was deduced from the following studies. To determine the terminal sequences the carboxymethyl (Cm)-ferredoxin (50 nmol) was subjected to manual Edman degradation to establish the amino (N)-terminal sequence of 21 residues. Carboxypeptidase A treatment of Cm-ferredoxin (5 nmol) released tyrosine (0.9 mol/mol protein) and leucine (1.0 mol/mol protein) over 30 min treatment at 40°, in conjunction with later data showing the carboxyl (*C*)-terminal sequence was -Leu-Tyr.

For subsequent sequence studies the Cm-ferredoxin (700 nmol) was treated with lysylendopeptidase (70 μg) in 0.1 M Tris–HCl buffer, pH 9, at 30° for 5 hr. The peptides were separated on a reverse-phase C18 column (TSK-GEL, ODS-120T, 4.6 × 250 mm) with a linear gradient of methyl cyanide from 0-40% using a Gilson HPLC system. The amino acid compositions of the resulting five peptides (K-1 to K-5) are given in Table 1; when summed there was good agreement to the composition data for the Cm-ferredoxin given the uncertainty inherent in information derived from 24 hr hydrolyses.

Each peptide was subjected to manual Edman degradation (K-1, 3 steps; K-2, 11 steps; K-3, 26 steps; K-4, 24 steps; and K-5, 8 steps). The sequence of the final peptide K-5 was therefore completely determined by this analysis.

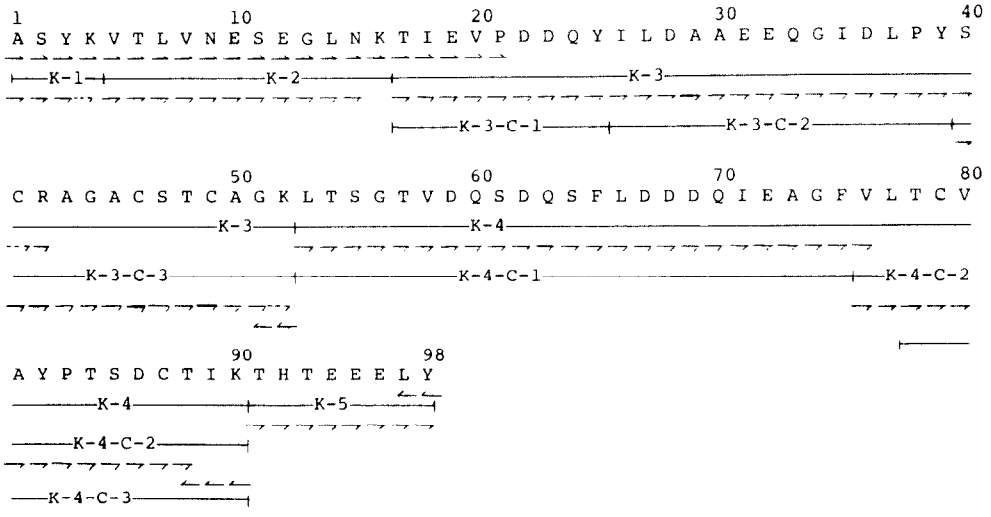

Fig. 2. Summary of the sequence determination for *Synechococcus* 6307 Cm-ferredoxin. K and C refer to lysylendopeptidase and chymotryptic peptides, respectively. Cys was identified as Cm-cysteine. The sequence was determined by a combination of manual Edman degradations of the intact protein (→ below sequence) and peptide fragments (→), and carboxypeptidase A and Y treatment (←). Dotted arrows represent ambiguous identifications. Single letter notation has been used for amino acids.

Table 1. Amino acid composition of Cm-ferredoxin and of the peptides derived by lysylendopeptidase digestion

Amino acid	Cm-Ferredoxin		Peptides (residue numbers in parentheses) K-1 (1-4)	K-2 (5-16)	K-3 (17-52)	K-4 (53-90)	K-5 (91-98)
Cmc	4.2	(5)		0.1	2.4 (3)	1.9 (2)	
Asp	12.4	(12)	0.1	2.0 (2)	4.4 (4)	6.0 (6)	0.1
Thr	10.0	(10)		1.1 (1)	2.1 (2)	4.6 (5)	1.9 (2)
Ser	7.5	(8)	1.0 (1)	1.0 (1)	2.0 (2)	3.5 (4)	0.1
Glu	15.1	(14)	0.1	2.1 (2)	5.8 (5)	4.7 (4)	3.1 (3)
Pro	4.0	(3)			1.7 (2)	1.1 (1)	
Gly	6.8	(6)	0.2	1.1 (1)	3.2 (3)	2.3 (2)	
Ala	7.7	(8)	1.0 (1)		4.9 (5)	2.4 (2)	
Val	5.3	(6)		1.8 (2)	1.1 (1)	2.6 (3)	
Ile	5.0	(5)			2.9 (3)	2.1 (2)	
Leu	7.5	(8)		1.9 (2)	2.3 (2)	3.2 (3)	1.0 (1)
Tyr	4.5	(5)	1.0 (1)		2.1 (2)	1.2 (1)	1.0 (1)
Phe	2.2	(2)			0.1	1.9 (2)	
Lys	3.6	(4)	1.1 (1)	0.9 (1)	1.0 (1)	1.0 (1)	0.1
His	1.0	(1)					0.9 (1)
Arg	0.9	(1)			1.0 (1)		
Total Residues		98	4	12	36	38	8
Yield (%)			42	44	31	37	44

Analyses were based on a 24 hr hydrolysis. The numbers in parentheses are those calculated from the final sequence.

Table 2. Amino acid composition of chymotryptic peptides K-3 and K-4

Amino acid	Peptides (residue numbers in parentheses) K-3-C-1 (17–25)	K-3-C-2 (26–39)	K-3-C-3 (40–52)	K-4-C-1 (53–75)	K-4-C-2 (76–90)	K-4-C-3 (78–90)
Cmc			3.3 (3)		2.0 (2)	2.0 (2)
Asp	1.9 (2)	2.1 (2)	0.1	4.7 (5)	1.1 (1)	1.1 (1)
Thr	0.9 (1)		1.1 (1)	1.8 (2)	2.7 (3)	2.6 (3)
Ser	0.1	0.1	2.1 (2)	2.7 (3)	1.0 (1)	1.0 (1)
Glu	2.1 (2)	3.3 (3)	0.1	4.1 (4)	0.2	0.2
Pro	n.d. (1)	n.d. (1)			n.d. (1)	n.d. (1)
Gly	0.1	1.0 (1)	2.1 (2)	2.1 (2)	0.2	0.3
Ala		1.6 (2)	2.5 (3)	1.0 (1)	1.0 (1)	1.0 (1)
Val	0.9 (1)			0.9 (1)	1.8 (2)	0.8 (1)
Ile	0.9 (1)	2.2 (2)		1.0 (1)	0.9 (1)	0.9 (1)
Leu		2.1 (2)		2.0 (2)	1.0 (1)	0.1
Tyr	0.9 (1)	0.8 (1)			0.8 (1)	0.8 (1)
Phe				1.9 (2)		
Lys			1.0 (1)	0.1	0.9 (1)	0.9 (1)
His						
Arg			1.1 (1)			
Total Residues	9	14	13	23	15	13
Yield (%)	69	69	40	23	27	22

Analyses were based on a 24 hr hydrolysis. The numbers in parentheses are those calculated from the final sequence.

n.d.; Not determined due to an instrumental fault.

Peptide K-3 (170 nmol) was further treated with chymotrypsin (1:100 w/w) in 0.01 M NH_4HCO_3, pH 8.0, at 40° for 3 hr and the resulting peptides purified by HPLC. The amino acid compositions of three peptides (K-3-C-1 to K-3-C-3) are given in Table 2. Subsequently, K-3-C-1 and K-3-C-2 could be placed in the sequence on this data alone given the information from the Edman degradation of K-3. Peptide K-3-C-3 was subjected to manual Edman degradation (40 nmol peptide) and carboxypeptidase Y (30 nmol peptide) to complete the sequence of K-3.

Similarly, peptide K-4 (210 nmol) was treated with chymotrypsin to give three peptides (K-4-C-1; K-4-C-2 and K-4-C-3). Peptide K-4-C-2 was subjected to manual Edman degradation (40 nmol peptide) and carboxypeptidase Y treatment (30 nmol peptide), respectively, and this information together with the amino acid compositions of the three peptides was sufficient to deduce the structure of K-4.

Overlapping peptides were not available to connect K-3 with K-4 and K-4 with K-5 but these fragments could be assigned in order with surety on the basis of the appreciable number of homologous sequences of [2Fe-2S] ferredoxins now documented [1, 3, 10].

So far, 19 complete sequences of cyanobacterial ferredoxins are available,together with a sequence for a putative ferredoxin in *Synechococcus* 6301 based on a gene sequence (Fig. 3). A comparison with these shows the features unique to *Synechococcus* 6307 ferredoxin are Ser-12 and Thr-95 which are not found in other cyanobacterial ferredoxins or those from higher plants or algae listed in [3]; subsequently the former has been found in *Ochromonas danica* ferredoxin (unpublished data). *Synechococcus* 6307 ferredoxin also lacks the deletions at positions 11 and 15 in the sequences as shown; these are found in ferredoxins from some unicellular cyanobacteria but are invariably lacking in those from filamentous species.

On the basis of the alignment for maximum homology, an amino acid difference matrix was compiled as shown in Fig. 4, in which one gap in the sequence was counted as one amino acid difference. Inspection shows that apart from clear relationship to ferredoxins from *Synechococcus* 6301 and 7942 (14 differences) the *Synechococcus* 6307 ferredoxin shows most resemblance to those from filamentous cyanobacteria, in particular *Mastigocladus laminosus* and *Chlorogloeopsis fritschii* where there are only nine and 14 differences, respectively. The ferredoxin corresponding to the gene sequences from *Synechococcus* 6301 is diverse from all the other ferredoxins and may represent a bound iron-sulphur centre [9]. The close relationship of *Synechococcus* 6307 to *Synechococcus* 6301 on the basis of their ferredoxins is deserving of comment. Their mol% GC are 71% and 55%, respectively, and on this basis they are sufficiently diverse to be placed in different subgroups of the genus [12]. Such a difference in mol% GC suggests unrelatedness at the genome level, though their ferredoxins, at least, are closely homologous and the two species are similar in structural and developmental respects.

The relationship of the cyanobacterial ferredoxins is emphasised in a phylogenetic tree (Fig. 5) constructed according to [13] on the basis of the aligned sequences. The matrix reconstructed from the branch lengths of the

```
                          1        20        40        60        80        100
(A) A. sacrum I           AS-YKVTLKT-PDG-DNVITVPDDEYILDVAEEEGLDLPYSCRAGACSTCAGKLVSGPA-PDEDQSFLDDDQIQAGYILTCVAYPTGDCVIETHKEEALY
(B) A. sacrum II          AT-YKVTLINEEEGINAILEVADDQTILDAGEEAGLDLPSSCRAGSCSTCAGKLVSGAAPNQDDQAFLDDDQLAAGWVMTCVAYPTGDCTIMTHQESEVL
(C) A. halophitica        AS-YKVTLINEEMGLNETIEVPDDEYILDVAEEEGIDLPYSCRAGACSTCAGKIKEGEI-DQSDQSFLDDDQIEAGYVLTCVAYPASDCTIITHQEEELY
(D) Synechococcus sp.     AT-YKVTLVR-PDGSETTIDVPEDEYILDVAEEQGLDLPFSCRAGACSTCAGKLLEGEV-DQSDQSFLDDDQIEKGFVLTCVAYPRSDCKILTNQEEELY
(E) Synechococcus 6301    AT-YKVTLVNAAEGLNTTIDVADDTYILDAAEEQGIDLPYSCRAGACSTCAGKVVSGTV-DQSDQSFLDDDQIAAGFVLTCVAYPTSDVTIETHKEEDLY
(F) Synechococcus 6301*   AT-YQVEVIY--QGQSQTFTADSDQSVLDSAQAAGVDLPASCLTGVCTTCAARILSGEV-DQPDAMGVGPEPAKQGYTLLCVAYPRSDLKIETHKEDELYALQFGQPG
(G) Synechococcus 7942*   AT-YKVTLVNAAEGLNTTIDVADDTYILDAAEEQGIDLPYSCRAGACSTCAGKVVSGTV-DQSDQSFLDDDQIAAGFVLTCVAYPTSDVTIETHKEEDLY
(H) Synechococcus 6307    AS-YKVTLVNESEGLNKTIEVPDDQYILDAAEEQGIDLPYSCRAGACSTCAGKLTSGTV-DQSDQSFLDDDQIEAGFVLTCVAYPTSDCTIKTHTEEELY
(I) Synechocystis 6714    AS-YTVKLIT-PDG-ENSIECSDDTYILDAAEEAGLDLPYSCRAGACSTCAGKITAGSV-DQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIETHKEEDLY
(J) Anabaena sp. 7120*    AT-FKVTLINEAEGTKHEIEVPDDEYILDAAEEQGYDLPFSCRAGACSTCAGKLVSGTV-DQSDQSFLDDDQIEAGYVLTCVAYPTSDVVIQTHKEEDLY
(K) An. variabilis        AT-FKVTLINEAEGTKHEIEVPDDEYILDAAEEEGYDLPFSCRAGACSTCAGKLVSGTV-DQSDQSFLDDDQIEAGYVLTCVAYPTSDCVIQTHKEEDLY
(L) An. variabilis 29413* AT-FKVTLINEAEGTSNTIDVPDDEYILDAAEEEGYDLPFSCRAGACSTCAGKLVSGTV-DQSDQSFLDDDQIEAGYVLTCVAYPTSDVTIQTHKEEDLY
(M) Ap. flos-aquae        AT-YKVTLI-DAEGTTTTIDCPDDTYILDAAEEAGLDLPYSCRAGACSTCAGKLVTGTI-DQSDQSFLDDDQVEAGYVLTCVAYPTSDVTIETHKEEDLY
(N) C. fritschii          AT-YKVTLINDAEGLNQTIEVDDDTYILDAAEEAGLDLPYSCRAGACSTCAGKIKSGTV-DQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIETHKEEELY
(O) Nostoc strain MAC I   ATVYKVTLV-DQEGTETTIDVPDDEYILDIAEDQGLDLPYSCRAGACSTCAGKIVSGTV-DQSDQSFLDDDQIEKGYVLTCVAYPTSDLKIETHKEEDLY
(P) Nostoc strain MAC II  AT-YKVRLFNAAEGLDETIEVPDDEYILDAAEEAGLDLPFSCRSGSCSSCNGILKKGTV-DQSDQNFLDDDQIAAGNVLTCVAYPTSNCEIETHREDAIA
(Q) N. muscorum           AT-FKVTLINEAEGTKHEIEVPDDEYILDAAEEEGYDLPFSCRAGACSTCAGKLVSGTV-DQSDQSFLDDDQIEAGYVLTCVAYPTSDVVIQTHKEEDLY
(R) M. laminosus          AT-YKVTLINEAEGLNKTIEVPDDQYILDAAEEAGIDLPYSCRAGACSTCAGKLISGTV-DQSDQSFLDDDQIEAGYVLTCVAYPTSDCVIETHKEEELY
(S) S. maxima             AT-YKVTLISEAEGINETIDCDDDTYILDAAEEAGLDLPYSCRAGACSTCAGKITSGSI-DQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIQTHQEEGLY
(T) S. platensis          AT-YKVTLINEAEGINETIDCDDDTYILDAAEEAGLDLPYSCRAGACSTCAGTITSGTI-DQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIKTHQEEGLY
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Fig. 3. Comparison of the amino acid sequences of cyanobacterial ferredoxins. Gaps are inserted to give maximum homology amongst the sequences. References for the sequences are: A–D, I, K, M–T as listed in [1]; E in [11]; F in [8]; G in [4, 5]; J in [6] and L in [7]. H is the present work. ★ represents a sequence derived from gene analysis.

A	Aphanothece sacrum I	0																		
B	Aphanothece sacrum II	38	0																	
C	Aphanothece halophitica	27	34	0																
D	Synechococcus sp.	29	41	23	0															
E	Synechococcus 6301	29	33	23	25	0														
F	Synechococcus 6301*	65	69	62	60	62	0													
G	Synechococcus 7942*	29	33	23	25	0	62	0												
H	Synechococcus 6307	28	31	16	23	14	63	14	0											
I	Synechocystis 6714	24	39	24	28	24	60	24	23	0										
J	Anabaena 7120*	27	34	23	26	17	63	17	18	25	0									
K	Anabaena variabilis	25	33	21	26	19	63	19	18	24	2	0								
L	Anabaena variabilis 29413*	26	34	21	25	15	61	15	18	24	6	6	0							
M	Aphanizomenon flos-aquae	28	35	24	27	15	61	15	22	20	17	18	14	0						
N	Chlorogloeopsis fritschii	27	30	16	26	13	55	13	14	17	17	16	16	14	0					
O	Nostoc MAC I	28	41	26	21	17	59	17	22	25	19	20	18	16	18	0				
P	Nostoc MAC II	37	38	32	35	28	67	28	29	35	29	28	29	31	25	34	0			
Q	Nostoc muscorum	26	34	22	27	18	63	18	19	25	1	1	5	17	17	20	29	0		
R	Mastigocladus laminosus	25	29	16	25	14	57	14	9	21	13	12	14	16	8	20	25	13	0	
S	Spirulina maxima	30	31	18	27	19	60	19	19	18	21	20	18	15	12	23	32	21	16	0
T	Spirulina platensis	31	31	18	28	18	60	18	17	20	21	20	18	15	11	23	30	21	15	4	0
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T

Fig. 4. Matrix of amino acid differences for cyanobacterial ferredoxins. The matrix was derived on the basis of the alignments given in Fig. 3 with each gap counted as one amino acid difference.

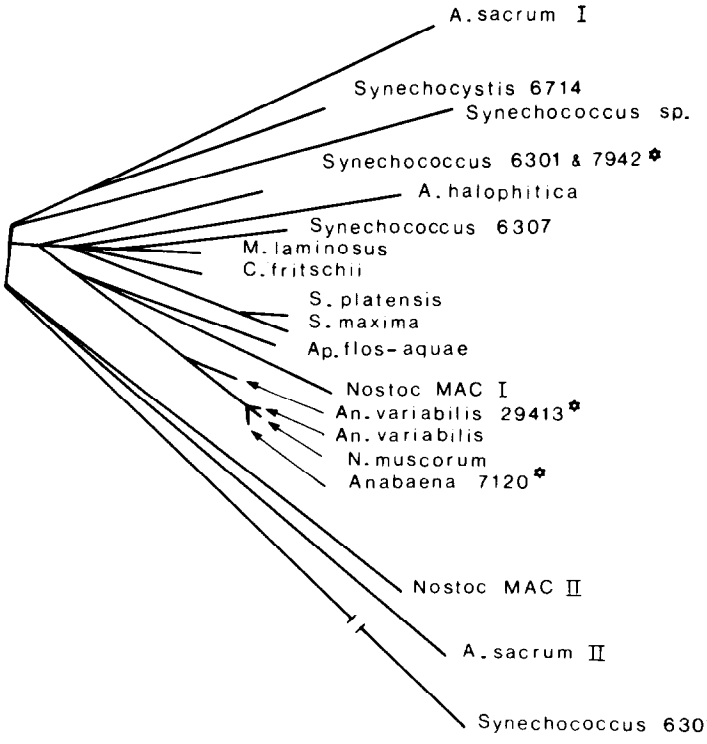

Fig. 5. A phylogenetic tree of cyanobacterial ferredoxins constructed according to [13].

tree was close to that made on the basis of the original amino acid differences with the average deviation between the two matrices being only 11.1%.

EXPERIMENTAL

Organism. Synechococcus PCC 6307 was obtained from the Paris Culture Collection (Pasteur Institute, Paris, France). Cultures were grown autotrophically in light [14]. Cultures at 30° were gassed continuously with air/CO_2 (19:1) during illumination (5000 lx). The cyanobacteria were grown in 8 l flasks with a 6% (v/v) inoculum. The yield from each flask was *ca* 15 g of cell paste; an Me_2CO- powder of the cells was prepared immediately and stored until use.

Isolation of ferredoxin. The procedure adopted for purification of the ferredoxin followed closely that described previously for the isolation of ferredoxin from another cyanobacterium *Nostoc* strain MAC [15].

Sequence determination. The amino acid sequence of the ferredoxin was determined by amino (*N*)-terminal manual Edman degradation of the carboxymethylated ferredoxin, carboxyl(*C*)-terminal analysis with carboxypeptidases A and Y, and manual Edman degradation of peptides derived by lysylendopeptidase treatment with supplementary sequencing using chymotrypsin. The experimental protocols have been described previously on a number of occasions [see e.g. 16].

Other methods were as in ref. [15], except mid-point redox potential determination [17].

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