CYANIDIUM CALDARIUM FERREDOXIN: A RED ALGAL TYPE?

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1. Introduction

Chloroplast-type ferredoxin is a 2 Fe–2 S iron–sulfur protein found in blue-green algae and photosynthetic eukaryotes [1]. A similar ferredoxin was also present in *Halobacterium* [2]. Recent studies on immunological cross reaction of ferredoxins showed the interrelations between blue-green and green algae or blue-green and red algae [3]. The amino acid sequences of ferredoxins from blue-green algae [4–9] and a green alga [10] have been determined, but no other algal ferredoxin sequence is available, although a partial sequence of a red alga, *Porphyra umbilicalis* is known [11]. The algal ferredoxins are evolutionarily a very diverse group judging from sequence comparisons [5,11].

We have purified a ferredoxin from an acidothermal alga, *Cyanidium caldarium* which is a unicellular eukaryotic organism of uncertain classification. This alga may represent the evolutionary transition from the prokaryotic blue-green algae to the simplest eukaryotic red alga [12]. We describe here the amino acid sequence of *Cyanidium caldarium* ferredoxin and compare it with other chloroplast-type ferredoxins to assess a taxonomic and evolutionary status of *Cyanidium*.

2. Materials and methods

2.1. *Mass production of* C. caldarium

C. caldarium Geitler em. Hirose (no. 1355/1 Allen) was from the Culture Collection of Algae and Protozoa, Cambridge. The mass production of this alga was performed in a 110 l algal pilot plant [13]. A semi-continuous culturing was performed. Every week 95 l of the algal suspension were harvested. Then an equal volume of fresh and sterile medium was added to the 15 l algal suspension remaining in the plant. Details of sterilization, inoculation and harvest were similar to those in [14]. On average, 750 g *Cyanidium* were harvested every 7 days.

2.2. *Purification of ferredoxin*

Wet cells, 1 kg, were homogenised with 20 mM Tris–HCl buffer (pH 7.5). The homogenate was sonicated for 30 s intervals for total of 10 min in an ice bath. Acetone at −15°C was slowly added to the sonicate to final conc. 30%. The mixture was centrifuged at 23 000 × *g* for 1 h. DE23 (Whatman DEAE-cellulose), 40 g, was stirred into the supernatant and the mixture let stand for 1 h. The DE23 was transferred to a small column (3.2 × 30 cm) and washed successively with 0.1 M and 0.2 M NaCl in buffer. The ferredoxin was eluted by 0.8 M NaCl in buffer. After adding ammonium sulphate to the eluate to 50%, the mixture was centrifuged and the supernatant containing the ferredoxin was dialysed against 20 l buffer overnight. Further purification of ferredoxin was carried out by standard procedures, viz. chromatography on DEAE-cellulose, Sephadex G-75 and hydroxylapatite [1].

2.3. *Sequence analyses*

About 1.3 μmol carboxymethyl (Cm)-ferredoxin

[6] were hydrolyzed with 0.3 mg trypsin for 2 h at 40°C in 1 ml 0.1 M Tris–HCl (pH 8.0). The digest was chromatographed on a Bio-Gel P-6 column (2 × 197 cm) by 0.2 M ammonium bicarbonate. Some fractions were further purified by paper electrophoresis, at pH 6.5. Staphylococcal protease (a gift of Dr R. Ambler, Edinburgh Univ.) was used to hydrolyze a large tryptic peptide T-3. The amino (N)- and carboxyl (C)-terminal sequences of Cm-ferredoxin and various peptides were determined by a manual Edman degradation [15] and a carboxypeptidase method [16,17], respectively. Phenylthiohydantoin derivatives of amino acids were identified by the thin-layer chromatography [18] or by paper electrophoresis, at pH 6.5 [19]. Amino acid compositions of CM-ferredoxin and peptides were determined by an amino acid analyzer (Beckman Model 120B) as usual [20] after 6 N HCl hydrolysis for 24 h or 72 h. Detailed procedures of sequence studies were essentially as in [2,6–8].

3. Results and discussion

Purified *Cyanidium* ferredoxin (5 mg/kg cells) showed similar optical absorption and electron paramagnetic spectra to those of other chloroplast-type ferredoxins. The absorbance ratio, $R=A_{420}/A_{280}$, was 0.52.

3.1. *Amino acid compositions and N- and C-terminal sequences of Cm-ferredoxin*

The amino acid composition of *C. caldarium* Cm-ferredoxin is shown in table 1. The total number of residues was 98. This composition agrees with that deduced from the sequence. Manual Edman degradation revealed the N-terminal sequence up to 14 residues without any ambiguity as shown in fig.1. Carboxypeptidase A released only leucine (1.00) and tyrosine (1.02) from Cm-ferredoxin after 15 min and no other residue was released after further digestion.

Table 1
Amino acid compositions of Cm-ferredoxin[a] and its tryptic peptides[b]

Cm-ferredoxin		T-1	T-2	T-3	T-4	T-5	T-6	Total
Lys	4.22(4)	1.09(1)	1.18(1)		1.05(1)	1.03(1)		4
His	1.71(2)		0.88(1)				1.12(1)	2
Arg	0.94(1)			0.88(1)				1
Cmc	5.31(5)			1.97(2)	1.89(2)		1.02(1)	5
Asp	12.7 (13)		1.06(1)	5.84(6)		5 00(5)	1.07(1)	13
Thr	5.54(6)			1.02(1)	1.01(1)		3.83(4)	6
Ser	6.92(7)	0.96(1)		1 09(1)	0 93(1)	1.81(2)	1.97(2)	7
Glu	15.2 (15)			6.88(7)		5.04(5)	3.08(3)	15
Pro	3.31(3)			1.99(2)			1.14(1)	3
Gly	6.29(6)			2.05(2)	1.88(2)	1.00(1)	1.03(1)	6
Ala	9.05(9)	0.98(1)		2.06(2)	2.90(3)		2.91(3)	9
Val	5.13(5)		1.02(1)			2.02(2)	1.97(2)	5
Ile	4.94(5)		0.82(1)	3.08(3)			0.98(1)	5
Leu	9.55(10)		1.05(1)	2.97(3)		2.84(3)	2.72(3)	10
Tyr	4.83(5)	0.96(1)		1.81(2)			1.96(2)	5
Phe	2.05(2)					1.01(1)	0.87(1)	2
Total	98	4	6	32	10	20	26	98
Yield (%)		80	45	96	95	94	63	

[a] Acid hydrolyses were carried out for 24 h and 72 h. The values of threonine and serine were obtained by extrapolation to 0 time hydrolysis. The values of valine and isoleucine were of 72 h hydrolysate

[b] The values were of 24 h hydrolysates without any corrections for incomplete hydrolysis or destruction

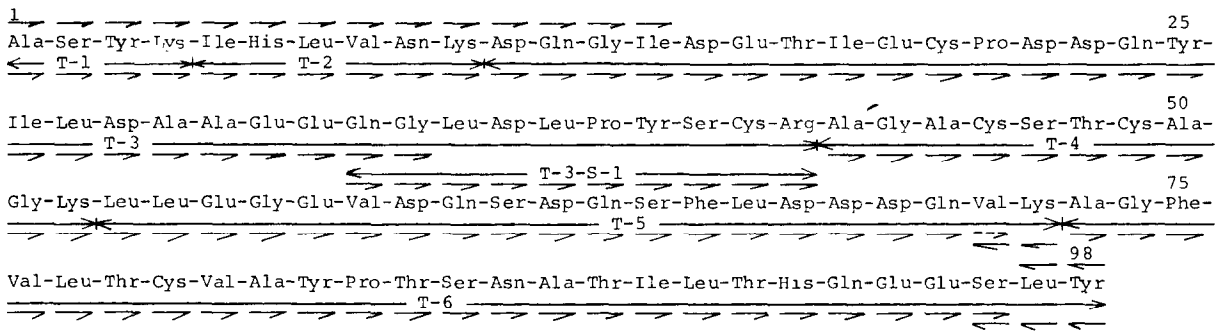

Fig.1. Summary of the sequence studies of *Cyanidium* ferredoxin. T- and S- refer, respectively, to peptides derived by tryptic digestion of Cm-ferredoxin and staphylococcal protease digestion of peptide T-3. Arrows (——→) above the sequence and below the sequences of peptides show, respectively, Edman degradations for Cm-ferredoxin and peptides. Arrows (←——) above and below the sequence show, respectively, carboxypeptidase A digestion on Cm-ferredoxin and peptides. A dotted arrow indicates an ambiguous identification.

3.2. *Tryptic peptides and reconstruction of the ferredoxin sequence*

Six peptides, T-1 to T-6, were obtained from the tryptic digest in good recovery (45–95%). The compositions and properties of the tryptic peptides are summarized in table 1. The sequence studies of these peptides are summarized in fig.1. Peptides T-1, T-2 and T-4 were completely sequenced by Edman degradation (T-1,4 steps; T-2, 6 steps; T-4, 10 steps). Large peptides T-3, T-5 and T-6 were partially sequenced by Edman degradations. Peptide T-3 was further digested with staphylococcal protease to give peptide T-3-S-1, C-terminal region of peptide T-3. Its amino acid composition was Arg, 0.92(1); Cm-cys, 0.89(1). Asp, 1.03(1); Ser, 0.86(1); Glu, 0.98(1), Pro, 0.92(1); Gly, 0.98(1); Leu, 1.80(2); Tyr, 0.85(1); and its sequence was determined by Edman degradation and gave the supplement of the unsequenced portion of peptide T-3. Carboxypeptidase digestions were performed on peptides T-5 and T-6 to obtain C-terminal sequences. Carboxypeptidase B digestion released lysine (1.09) from peptide T-5 after 1 h and further digestion with carboxypeptidase A released valine (1.05) after 1 h. Carboxypeptidase Y digestion of peptide T-6 released leucine (0.40) and tyrosine (0.73) after 5 min and leucine (0.84), tyrosine (0.87) and serine (0.47) after 1 h. Thus, C-terminal sequences of peptides T-5 and T-6 were determined as –Val–Lys and –Ser–Leu–Tyr, respectively.

N-terminal sequence of Cm-ferredoxin gave the overlaps of peptides T-1, T-2 and T-3. No effort to overlap peptides T-4 and T-5 was made and they were aligned on the basis of homology to all other chloroplast-type ferredoxins. Peptide T-6 was C-terminal peptide. These results gave the complete sequence of *C. caldarium* ferredoxin (fig.1) and mol. wt 10 695 excluding iron and sulfur atoms.

3.3. *Comparison of sequences of* Cyanidium *and other ferredoxins*

Sequence of *Cyanidium* ferredoxin is compared with those of spinach, *Scenedesmus quadricauda* (a green alga), *Nostoc muscorum* (a blue-green alga), and *Porphyra umbilicalis* (a red alga) (fig.2). There are 37, 27, and 28 amino acid differences between *Cyanidium* and spinach, *Scenedesmus,* and *Nostoc* ferredoxins, respectively. From the number of amino acid differences, *Cyanidium* ferredoxin shows closer similarity to algal ferredoxins than to spinach ferredoxin. *Cyanidium* ferredoxin has two extra residues, lysine and isoleucine at positions 10 and 14, respectively. From the data available at present time all blue-green algal ferredoxins except for *Aphanothece sacrum* ferredoxin I [6] has two insertions at these positions, although the amino acid residues are different. Recently a partial sequence of *Porphyra* ferredoxin was reported and this ferredoxin had also such insertions [11]. In terms of the presence of these insertions *Cyanidium* ferredoxin seems to be more closely related to blue-green and red algal ferredoxins. *Cyanidium caldarium* is a unicellular eukaryotic alga of uncertain classification. In the

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    1                                                                  50
(a) A A Y K V T L V T - P T G - N V E F Q C P D D V Y I L D A A E E E G I D L P Y S C R A G S C S S C A
(b) A T Y K V T L K T - P S G - D Q T I E C P D D T Y I L D A A E E A G L D L P Y S C R A G A C S S C A
(c) A T F K V T L I N E A E G T K H E I E V P D D E Y I L D A A E E E G Y D L P F S C R A G A C S T C A
(d) A S Y K I H L V N K D Q G I D E T I E C P D D Q Y I L D A A E E Q G L D L P Y S C R A G A C S T C A
(e) A D Y K I H L V S K E E G I D V T F D C S E D T Y I L D A A E E E G I E L

    51                                                                 99
(a) G K L K T G S L N Q D D Q S F L D D D Q I D E G W V L T C A A Y P V S D V T I E T H K E E D L T A
(b) G K V E A G T V D Q S D Q S F L D D S Q M D G G F V L T C V A Y P T S D C T I A T H K E E D L F
(c) G K L V S G T V D Q S D Q S F L D D D Q I E A G Y V L T C V A Y P T S D V V I Q T H K E E D L Y
(d) G K L L E G E V D Q S D Q S F L D D D Q V K A G F V L T C V A Y P T S N A T I L T H Q E E S L Y
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Fig.2. Comparison of several representative chloroplast-type ferredoxins: (a) spinach [1]. (b) *Scenedesmus quadricauda* [10]; (c) *Nostoc muscorum* [7]. (d) *Cyanidium caldarium*, here: (e) *Porphyra umbilicalis* [11].

sequence comparison of 19 ferredoxins unique amino acid residues occupy several positions in *Cyanidium* and in the partial sequence of *Porphyra* ferredoxins, such as, Ile-5, His-6 and Lys-10. This suggests *C. caldarium* may be a member of red algae. However, more complete and extensive sequence study of red algal ferredoxins is required to confirm that *Cyanidium* forms an evolutionary link between the blue-green and red algae.

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Addendum

During the preparation of this manuscript we were notified by Dr L. J. Rogers that *P. umbilicalis* ferredoxin sequence has been completed.

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