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COMPARATIVE PROPERTIES OF FERREDOXINS FROM A MARINE AND FRESHWATER SPECIES OF *PORPHYRIDIUM*

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Abstract—Ferredoxins were isolated from the freshwater red alga *Porphyridium aerugineum*, and from *Porphyridium cruentum*, a related marine species. A single two-iron ferredoxin of *ca* molecular weight 11 000 was present in both. The midpoint potentials of the ferredoxins were close to −400 mV and they showed the same activity in supporting pyruvate decarboxylation by the phosphoroclastic system of *Clostridium pasteurianum* or $NADP^+$ photoreduction by chloroplasts. Close similarity of the ferredoxins was substantiated by *N*-terminal amino acid sequence determination; there were only three differences between the sequences of the first 33 residues. The *N*-terminal sequences were more closely related to that of the ferredoxin from *Porphyra umbilicalis*, also in the Bangiophycidae, than to ferredoxin from *Rhodymenia palmata*, which is placed in the subclass Florideophycidae of the Rhodophyceae.

INTRODUCTION

Ferredoxins are small electron-transfer proteins which function in oxidation–reduction reactions in a wide range of organisms [1]. A crucial role in photosynthetic organisms is in coupling electron flow from photosystem 1 to $NADP^+$ and amongst the reasons for the current interest in ferredoxins is to establish structure–function relationships and to gain an insight into the biochemical evolution of photosynthesis. Within particular groups of organisms amino acid sequence data might also give information on phylogenetic relationships.

Algal ferredoxins from only a few species have been studied in detail, and amino acid sequence information is available only for the ferredoxins from the green algae *Dunaliella salina* [2] and *Scenedesmus quadricauda* [3], the red algae *Porphyra umbilicalis* [4] and *Rhodymenia palmata* [5], and *Cyanidium caldarium* [6], which is of uncertain taxonomic position. Studies on ferredoxins from two other red algae, *Porphyridium aerugineum* and *Porphyridium cruentum*, in the genus *Porphyridiacea* [7] are now reported. These are of particular interest from a comparative viewpoint since whereas *Porphyridium cruentum*, like the great majority of red algae, is a marine species the related red alga *Porphyridium aerugineum*, which is blue-green in colour since it lacks phycoerythrin, inhabits a freshwater environment [8].

RESULTS AND DISCUSSION

Homogeneity of the ferredoxins

Analytical polyacrylamide gel electrophoresis of *Porphyridium cruentum* and *P. aerugineum* ferredoxins in 10, 15 and 20% (w/v) gels showed a single stained band; in the lowest concentration gel the ferredoxins migrated at the anion front. As with the other red algae so far studied [5, 9], there was only one ferredoxin present in each *Porphyridium* species.

Composition and physical properties

The properties of the ferredoxins are summarized in Table 1. The absorption spectra showed slight differences in the visible region and in this respect the spectrum for *Porphyridium aerugineum* ferredoxin was virtually identical to that of *Rhodymenia palmata* ferredoxin [5] while the spectrum for *Porphyridium cruentum* ferredoxin was the same as that for the ferredoxin from *Porphyra umbilicalis* [9]. The relative extinction at higher wavelengths compared to 276 nm was lower for *Porphyridium cruentum* ferredoxin than for the other red algal ferredoxins. On treating the *Porphyridium* ferredoxins with dithionite, the extinction at 420 nm decreased by about 40% [1]. The molar absorption coefficients at 423 nm were estimated on the basis of both protein and iron determinations; the values of approximately 5.7 l./mmol/cm are similar to those for other red algal [5] and cyanobacterial [10, 11] ferredoxins, though higher values are obtained for the ferredoxins from higher plants (see e.g. [12]).

Analyses of non-haem iron and labile sulphur showed that both *Porphyridium* ferredoxins were of the [2Fe–2S] type. However, in contrast to previous studies of other algal or cyanobacterial ferredoxins, successful titration of labile sulphur in *Porphyridium cruentum* ferredoxin required a pre-incubation with alkaline zinc acetate [13]; this method was then also used with *Porphyridium aerugineum* ferredoxin.

The MWs of the ferredoxins were close to 12 000 by meniscus-depletion and low-speed equilibrium sedimentation. The partial specific volumes used in the calculations (*Porphyridium cruentum*, 0.713; *Porphyridium*

Table 1. Properties of the ferredoxins from *Porphyridium aerugineum* and *Porphyridium cruentum*

	Porphyridium aerugineum	*Porphyridium cruentum*
Absorption maxima UV (nm)	276, sh 281	276, sh 281
visible (nm)	329, 423, 465	326, 420, 464
Ratio A_{max}/A_{276}	0.76, 0.56, 0.51	0.53, 0.43, 0.39
Log ε 423 nm	(3.75)	(3.76)
Non-haem Fe (atoms/molecule)	1.9 ± 0.1; $n = 4$	1.8 ± 0.1; $n = 4$
Labile sulphur (atoms/molecule)	1.7; $n = 2$	1.8 ± 0.2; $n = 4$
Total sulphur* (atoms/molecule)	(3)	7
MW		
By ultracentrifugation	11 700; $n = 2$	$11\,900 \pm 100$; $n = 3$
From amino acid composition	10 700	10 400
Activity†		
NADP photoreduction	31.6	32.1
Phosphoroclastic reaction	11.8	12.4
Midpoint redox potential (mV) at pH 8.0	−394	−405

Some values are given as means ±s.d. for n experiments.

* From ΔA_{250} after reaction with excess of *p*-hydroxymercuribenzoate.

† μmol $NADP^+$ reduced/mg of chlorophyll/hr and μmol acetyl-phosphate formed/hr, respectively.

aerugineum, 0.719) were derived from amino acid composition studies and are possibly somewhat high [14] because of the influence of the iron–sulphur centre in the native protein; MWs estimated from amino acid composition are somewhat lower. Nevertheless, the *Porphyridium* ferredoxins are clearly the same size as those from plants, other algae, and nearly all cyanobacteria.

The ferredoxins both gave a rhombic e.p.r. signal in the reduced state characteristic of two-iron ferredoxins [1], and in potentiometric titrations both were shown to be one-electron acceptors with midpoint potentials close to −400 mV. Unlike the cyanobacterial ferredoxins [15], redox potentials of red algal ferredoxins appear to fall within a narrow range.

The *Porphyridium* ferredoxins differed in amino acid composition (Table 2); in particular *Porphyridium cruentum* ferredoxin contained appreciably more threonine and serine, and less lysine, than the ferredoxin from *Porphyridium aerugineum*. The latter contained a single methionine and two histidines, like the ferredoxin from *Porphyra umbilicalis* [9], whereas *Porphyridium cruentum* ferredoxin is similar to *Rhodymenia palmata* ferredoxin [5] in that methionine is absent and there is only a single histidine. However, in some other respects, e.g. presence of two arginine residues, the *Porphyridium* ferredoxins differ from those from the other two red algae. Determination of cysteine in *Porphyridium aerugineum* by titration with *p*-chloromercuribenzoate gave particular

Table 2. Amino acid compositions of *Porphyridium* ferredoxins

	Content (residues/molecule)				
	P. aerugineum	*P. cruentum*		*P. aerugineum*	*P. cruentum*
Asx	12	12	Met	1	0
Thr	6	9	Ile	5	5
Ser	7	9	Leu	8	8
Glx	12	12	Phe	2	2
Pro	3	2	Lys	5	2
Gly	7	8	Tyr	4	4
Ala	11	11	His	2	1
Val	7	7	Arg	2	2
½Cys	4*	5	Trp	0	0

Values, based on three separate analyses, are given as the nearest integer for the minimum molecular weight.

Cysteine was determined by titration with *p*-hydroxymercuribenzoate, and tryptophan by a spectrophotometric method.

* Assumed, because of difficulty in determination (see Table 1).

difficulty, though this method was used successfully for *Porphyridium cruentum* ferredoxin and for other red algal [5,9] and cyanobacterial [10,11] ferredoxins. It is assumed (Table 2) that *Porphyridium aerugineum* ferredoxin will nevertheless possess at least four cysteines; these are required to bind the [2Fe–2S] active centre.

Biochemical properties

The *Porphyridium* ferredoxins both supported $NADP^+$ photoreduction by barley chloroplasts depleted of native ferredoxin at a rate of about 32 μmol $NADP^+$ photoreduced/hr per mg of chlorophyll which was only slightly less than that for *Porphyra umbilicalis* ferredoxin in parallel experiments. Their ability to support pyruvate decarboxylation by the phosphoroclastic system of *Clostridium pasteurianum* were also the same within experimental error. In terms of maximum activity, the *Porphyridium* ferredoxins approached the efficiency of the native two-cluster-eight-iron ferredoxin; however, the K_ms for the red algal ferredoxins were close to 4.2×10^{-5} M, *ca* ten-fold higher than the $K_{m(Fd)}$ for *Clostridium pasteurianum* ferredoxin. In these experiments the *Porphyridium* ferredoxins were rather more active than *Porphyra umbilicalis* ferredoxin and appreciably more active than that from *Rhodymenia palmata.*

N-*Terminal amino acid sequences*

The *N*-terminal sequences (Fig. 1) of the *Porphyridium* ferredoxins were determined by using single automatic sequence analysis on *ca* 500 nmol of carboxymethylated protein. With *Porphyridium aerugineum* ferredoxin, identification of all residues was possible, though at position 21 the evidence for serine instead of cysteine was not as strong as we would have liked in view of the homology of cysteine (or valine) at this position in other two-iron ferredoxins. However, the ferredoxin from another red alga, *Rhodymenia palmata*, possesses glycine instead of cysteine or valine at this position. In *Porphyridium cruentum* ferredoxin there was some doubt about position 7 and the acidic residues at positions 24, 29, 32 and 33. *Porphyridium aerugineum* and *Porphyridium cruentum* ferredoxins show a close resemblance with only three amino acid differences in the first 33 positions.

The amino acid sequences are compared in Table 3 to those for the corresponding sequences of ferredoxins from two other red algae, two green algae and *Cyanidium caldarium*; within the algae only *Dunaliella salina* produces two ferredoxins. Compared to these other algal ferredoxins, and the ferredoxins from plants and cyanobacteria, a notable feature of *Porphyridium aerugineum* ferredoxin is the additional residue at position 3. This also occurs in *Rhodymenia palmata* ferredoxin, where lysine is also found in this position. Although an insertion also occurs here in ferredoxin I from *Nostoc* strain MAC, in that case it is one of the two additional residues apparently present when proline at position 12 is absent [10]. Both *Porphyridium* ferredoxins lack this proline and possess additional residues at positions 11 (glutamic acid) and 15 (isoleucine). They are also the only two-iron ferredoxins to have arginine at position 7, while valine at position 25 is only found elsewhere in the higher plant ferredoxins [2].

Within the algal ferredoxins the *Porphyridium* ferredoxins show greatest resemblance (Table 4) to *Porphyra umbilicalis* ferredoxin; resemblance to *Cyanidium* and *Scenedesmus* ferredoxins is also marked. When other two-iron ferredoxins are considered this group of red algal ferredoxins shows good similarity (6–10 differences) with *Spirulina maxima* and *Spirulina platensis* ferredoxins, a conclusion confirmed by consideration of the full sequences of some of these ferredoxins [2]. In a study of the comparative immunochemistry of a range of bacterial, algal and plant ferredoxins [16], ferredoxins from *Cyanidium caldarium* and *Porphyra umbilicalis* reacted well with antibody to *Spirulina maxima* ferredoxin.

In contrast, on the basis of the present limited sequence data the ferredoxin from *Rhodymenia palmata* is very different to the other red algal ferredoxins and appears closer to the green algal ferredoxins, though the resemblance is not strong; it belongs to the subclass Florideophycidae. Two other red algae in this subclass, *Chondrus crispus* and *Gigartina stellata*, produce negligible amounts of ferredoxin [17].

The resemblance in amino acid sequence of *Cyanidium caldarium* ferredoxin to the ferredoxins of *Porphyridium cruentum* and *Porphyra umbilicalis* is of particular interest. *Cyanidium caldarium* is of uncertain taxonomic position, but is placed by some authorities in the red algae, subclass Bangiophycidae, in the same order Porphyridiales as *Porphyridium aerugineum* and *Porphyridium cruentum* [7]. This placement is supported by the similarity of their ferredoxins.

EXPERIMENTAL

Algal strains. Porphyridium aerugineum Geitler (Pringsheim strain) and *Porphyridium cruentum* Nägeli (Vischer strain) were obtained from the Culture Centre of Algae and Protozoa, Cambridge, U.K. Cultures were maintained at 20° in dim light in liquid medium, *P. cruentum* as in ref. [18] and *P. aerugineum* as in ref. [19] except medium PII was replaced by microelement soln [20], also containing 0.13 μg EDTA · Na_2.

Growth of organisms. The growth medium contained (per l.): NaCl, 27.0 g; $MgSO_4 \cdot 7H_2O$, 6.6 g; $MgCl_2 \cdot 6H_2O$, 5.6 g; $CaCl_2 \cdot 2H_2O$, 1.5 g; KNO_3, 1.0 g; KH_2PO_4, 0.07 g; $NaHCO_3$, 0.04 g; 1.0 ml microelement soln [20] also containing 0.13 μg EDTA · Na_2, and 1.0 ml ferric citrate soln [21]; buffered to pH 7.6 by 20 ml 1 M Tris (Sigma 7–9). Organisms were grown in 800 ml medium in Roux bottles incubated in environmental

	1			5				10				15	
P. aerugineum	Ala	Lys	Lys	Tyr	Lys	Val	Arg	Leu	Leu	Ser	Glu	Ala	Glu	Gly	Ile
P. cruentum	Ala	Thr	–	Tyr	Lys	Val	(Arg)	Leu	Leu	Ser	Glu	Ala	Glu	Gly	Ile

	16			20				25				30	
	Asp	Val	Thr	Ile	Asp	(Ser)	Ala	Asp	Asp	Val	Tyr	Ile	Leu	Asp	Ala
	Asp	Val	Thr	Ile	Asp	Cys	Ala	Asp	(Asp)	Val	Tyr	Ile	Leu	(Asp)	Ala

Fig. 1. *N*-Terminal amino acid sequences of *Porphyridium aerugineum* and *Porphyridium cruentum* ferredoxins. Residues 31–40 for *Porphyridium aerugineum* were Ala Glu Glu Gln Gly Ile Asp Leu Pro Tyr.

Table 3. Comparison of the *N*-terminal amino acid sequences of ferredoxins from two *Porphyridium* species with those of other eukaryotic algae

					5					10					15					20					25					30			
Scenedesmus quadricauda	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
Dunaliella salina I	—	S	—	Y	M	V	T	L	K	T	—	P	S	G	—	E	Q	K	V	E	V	S	P	D	S	Y	I	L	D	A	A	E	E
Dunaliella salina II	A	—	—	Y	K	V	T	L	K	T	—	P	S	G	—	D	Q	T	I	E	V	S	P	D	A	Y	I	L	D	A	A	E	E
Porphyra umbilicalis	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
Rhodymenia palmata	A	V	K	Y	T	V	T	L	S	T	—	P	G	G	—	V	E	E	I	E	G	D	E	T	S	Y	V	L	D	S	A	E	D
Porphyridium aerugineum	A	K	K	Y	K	V	R	L	L	S	E	A	E	G	I	D	V	T	I	D	(S)	A	D	D	V	Y	I	L	D	A	A	E	E
Porphyridium cruentum	A	T	—	Y	K	V	(R)	L	L	S	E	A	E	G	I	D	V	T	I	D	C	A	D	(D)	V	Y	I	L	(D)	A	A	(E)	(E)
Cyanidium caldarium	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

The sequences are taken from ref. [2] except *Rhodymenia palmata* [5]. The sequences were aligned to give maximum similarity. Residues in parentheses are less certain.

Table 4. Matrix of amino acid sequence differences for algal ferredoxins; I and II refer to two different types. Based on first 33 positions

		(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
Cyanidium caldarium	(a)								
Dunaliella salina I	(b)	18							
Dunaliella salina II	(c)	14	7						
Scenedesmus quadricauda	(d)	11	10	5					
Porphyridium aerugineum	(e)	14	20	15	14				
Porphyridium cruentum	(f)	12	19	14	11	3			
Porphyra umbilicalis	(g)	10	19	15	14	12	10		
Rhodymenia palmata	(h)	21	17	16	16	21	22	23	

The sequences for (a)–(d) are taken from [2]; for (g) from [4] and (h) from [5].

cabinets maintained at 20° and illuminated (4300 lx) by fluorescent lights. Cultures were gassed with air–CO_2 (19:1) and were stirred. After *ca* 6 days 400 ml culture was inoculated into 8 l. of fresh medium in 10 l. flasks and incubations continued under the same conditions with a gas flow of 1 l./min. Growth was followed by measuring the turbidity at 730 nm using a Klett–Summerson colorimeter with Kodak Wratten 88A filter. Cultures were harvested at late log phase, about 8 days' growth, using an Alfa-Laval LAB 102B continuous flow centrifuge (Alfa Laval Co., Brentford); organisms were washed with 150 mM Tris–HCl, pH 7.4. The approximate yields from a total of 64 l. culture were 500 g wet wt cells.

Preparation of Me_2CO*-dried powders.* Cells were suspended in 3 vol. distilled water at 4° and added slowly to 16 vol. vigorously stirred Me_2CO at −20°. The suspension was stirred for 1 hr at 4° and then filtered through a sintered glass funnel under slight vacuum. The powder was washed with Me_2CO at −20° until the filtrate was colourless and then dried and stored in a vacuum desiccator over $CaCl_2$; *ca* 60 g of Me_2CO-dried powder were obtained from 500 g wet wt algal cells.

Isolation of ferredoxins. All stages of the purification were carried out in a cold room at 4° unless stated otherwise. Approximately 125 g Me_2CO-dried powder were homogenized in 2.5 l. 150 mM Tris–HCl, pH 7.4, and the suspension left for 15 hr before centrifuging at 13 000 *g* for 60 min (*Porphyridium cruentum*) or 50 000 *g* for 30 min (*Porphyridium aerugineum*) the supernatant being retained. To this was added, in the case of *Porphyridium cruentum*, 70 g DEAE-cellulose (Whatman DE-52) previously equilibrated against 150 mM Tris–HCl, pH 7.4. The *Porphyridium aerugineum* extract was very viscous and was left for 1 hr at 20° after addition of 2 mg each of deoxyribonuclease and ribonuclease to degrade polynucleotides. This gave some decrease in viscosity but it was still necessary to dilute the extract to 4.5 l. with buffer before addition of 100 g DEAE-cellulose. After 3 hr the resin in each case was recovered by centrifugation at 5000 *g* for 10 min and washed in 1 l. 150 mM Tris buffer before resuspension in 150 ml 300 mM Tris–HCl buffer, pH 7.4, containing 550 mM NaCl. After centrifugation the supernatant was retained and left for 2 hr at 20° after addition of 1 mg each of deoxyribonuclease and ribonuclease before adjustment at 4° to 60% saturation with $(NH_4)_2SO_4$. Anion-exchange chromatography was carried out on a column of DEAE-cellulose (20 cm × 1.5 cm). The resin had been equilibrated with 150 mM Tris–HCl buffer, pH 7.4, and the supernatant obtained by centrifugation at 40 000 *g* for 60 min after $(NH_4)_2SO_4$ treatment was dialysed for 15 hr against the same buffer before it was applied to the column. The column was developed successively with 300 ml 150 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl, and 500 ml of the same buffer containing 0.2 M NaCl. The elution vol. of the single ferredoxin present in each case was approximately 450 ml. Fractions with A_{423}/A_{276} ratios greater than 0.1, *Porphyridium cruentum*; 0.15, *Porphyridium aerugineum* were pooled and separately concd by using small (6 cm × 1 cm) DEAE-cellulose columns. Subsequent gel filtration on a column of Sephadex G-50 (35 cm × 2.5 cm) was as in ref. [10]. Fractions containing A_{423}/A_{276} ratios greater than 0.15, *Porphyridium cruentum*; 0.35, *Porphyridium aerugineum*, were pooled and concd by using a small DEAE-cellulose column before storage at −20° or immediate use. Yields of ferredoxin were *Porphyridium cruentum*, 14 mg and *Porphyridium aerugineum*, 24 mg.

Methods. These were described elsewhere [10] except for the determination of labile sulphur which was as described in [13].

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