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PARTIAL STRUCTURE AND PROPERTIES OF THE FERREDOXIN FROM *RHODYMENIA PALMATA*

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Key Word Index—*Rhodymenia palmata*; Rhodophyta; red algae; ferredoxin; amino acid sequence.

Abstract—A ferredoxin of MW 11 000 was isolated from the marine alga *Rhodymenia palmata* (*Palmaria palmata*). In its oxidised form the ferredoxin had absorption maxima at 276, sh 281, 328, 423 and 465 nm and contained a single [2Fe–2S] cluster. The midpoint potential of the ferredoxin was −400 mV and it effectively mediated electron transport in $NADP^+$-photoreduction by higher plant chloroplasts, and pyruvate decarboxylation by the phosphoroclastic system of an anaerobic bacterium. The amino acid composition was Lys_3, His_1, Arg_1, Asx_{12}, Thr_9, Ser_8, Glx_{13}, Pro_4, Gly_8, Ala_7, Cys_5, Val_8, Ile_4, Leu_9, Tyr_4, Phe_2; tryptophan and methionine were absent from the molecule. The *N*-terminal amino acid region consisting of *ca* half the total amino acid sequence was determined using an automatic sequencer.

INTRODUCTION

Ferredoxins are possibly the simplest electron-transfer proteins and are being extensively studied to establish both structure–function and phylogenetic relationships (see refs. [1 and 2]). However, excluding studies with cyanobacteria (Cyanophyceae; blue-green algae) there have been few detailed studies on the ferredoxins of algae, though such information might give valuable insight on phylogenetic relationships and into the biochemical evolution of photosynthesis. Amongst the algae from which ferredoxin has been isolated are: in the Euglenophyceae, *Euglena gracilis* [3]; in the Chlorophyceae, *Chlamydomonas reinhardi* [3], *Dunaliella salina* [2], and *Scenedesmus quadricauda* [4]; in the Xanthophyceae, *Botrydiopsis alpina* [5] and *Bumilleriopsis filiformis* [6]; in the Rhodophyceae, *Porphyra tenera* [7], *Porphyra umbilicalis* [8, 9], and *Porphyridium cruentum* [5]; and of anomalous taxonomic position, *Cyanidium caldarium* [10]. A number of these are however preliminary reports and so far only the ferredoxins from the phytoflagellate *Euglena gracilis*, the yellow-green alga *Bumilleriopsis filiformis*, the red alga *Porphyra umbilicalis*, the green algae *Dunaliella salina* and *Scenedesmus quadricauda*, and *Cyanidium caldarium* have been studied in detail; the amino acid sequences of ferredoxins from the last four species have been determined.

We now report investigations on the ferredoxin from *Rhodymenia palmata* (L.) Greville, alternatively named *Palmaria palmata* (L.) Stackhouse [11, 12], a member of the genus *Palmaria* in the Rhodophyta. This is a member of the family Florideophyceae within the Rhodophyta whereas *Porphyra umbilicalis*, the only other red alga for which comparable data have been reported, is a member of the Bangiophyceae.

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RESULTS AND DISCUSSION

Homogeneity of the ferredoxin

Analytical polyacrylamide gel electrophoresis of *Rhodymenia palmata* ferredoxin on 10, 15 and 20% (w/v) gels showed a major heavily stained band; in the lower concentration gels the ferredoxin migrated at the anion front. A single minor component of molecular weight *ca* 21 000 was also present as indicated by SDS-polyacrylamide gel electrophoresis. This second band was absent if β-mercaptoethanol were also present in the buffers used for electrophoretic studies suggesting it to be due to a dimeric ferredoxin formed by intermolecular disulphide bridging. Dimerisation of [2Fe–2S] ferredoxins from *Cucurbita pepo* [13], *Medicago sativa* [14] and *Spinacia oleracea* [15] have been previously reported.

Composition and physical properties

Some of the properties of *Rhodymenia palmata* ferredoxin are summarized in Table 1. The visible- and ultraviolet-absorption spectrum was similar to that reported for other algal ferredoxins, including that from *Porphyra umbilicalis* [8]; the relative absorbances for the main peaks were also similar. The molar extinction coefficient at 423 nm, based on determination of iron, and checked by determination of protein, was 5.8 l./mmol/cm. This is similar to that for *Porphyra umbilicalis* ferredoxin and for the ferredoxins from the cyanobacterium *Nostoc* strain MAC [16], though appreciably lower than that found for the ferredoxins from *Pisum sativum* [17] and that quoted for *Spinacia oleracea* ferredoxin [18]. These differences may be a reflection of differing environments of the iron–sulphur chromophore in different ferredoxins.

Analyses of non-haem iron and labile sulphur left no doubt that *Rhodymenia palmata* ferredoxin possessed a single [2Fe–2S] cluster, in common with all algal or cyanobacterial ferredoxins so far studied. This was

Table 1. Properties of *Rhodymenia palmata* ferredoxin

Absorption maxima (nm)	276, sh 281, 328, 423, 465
Ratio A_{max}/A_{276}	0.73, 0.57, 0.53
Log ε 423 nm	(3.77)
Non-haem iron (atoms/molecule)	$1.8 \pm 0.3; n = 4$
Labile sulphur (atoms/molecule)	$1.7 \pm 0.2; n = 4$
Total sulphur (atoms/molecule)	7
MW	
By ultracentrifugation	12100; $n = 2$
From amino acid composition	10800
Activity	
$NADP^+$ photoreduction (μmol $NADP^+$/mg of chl./hr)*	45
Phosphoroclastic reaction (μmol acetyl-P formed/hr)†	8.5
Midpoint redox potential (mV)‡	−402

* By barley chloroplasts depleted of native ferredoxin. † By *C. pasteurianum* extracts depleted of native ferredoxin. ‡ Value from [19]. Some values are given as means ± s.d. for *n* experiments.

substantiated by the rhombic e.p.r. signal given by reduced ferredoxin, which titrated as a one-electron acceptor, with a midpoint redox potential of −400 mV, close to that for *Porphyra umbilicalis* ferredoxin. There is no evidence as yet for diversity in redox potential in algal ferredoxins, such as found in those from cyanobacteria [19].

The amino acid composition of *Rhodymenia palmata* ferredoxin based on 24 hr hydrolyses with valine and isoleucine confirmed by analyses at 48 and 72 hr, was Asx_{12}, Thr_9, Ser_8, Glx_{13}, Pro_4, Gly_8, Ala_7, Val_8, Cys_5, Ile_4, Leu_9, Phe_2, Lys_3, Tyr_4, His_1, Arg_1. Methionine and tryptophan were both absent, the latter as determined by a spectrophotometric method. Both are usually absent from algal and cyanobacterial ferredoxins, though a single methionine is present in *Porphyra umbilicalis* ferredoxin [8], and ferredoxin II of *Aphanothece sacrum* contains two methionine and one tryptophan residue [20]. Cysteine was determined separately in the native protein by titration with *p*-hydroxymercuribenzoate, which indicated that 9 mol of reagent reacted with each mol of ferredoxin, indicating that five cysteine residues were present since the two labile sulphur groups of the [2Fe–2S] cluster would each react with two molecules of reagent.

The total of some 98 amino acids, with 2Fe + 2S, gives a minimum MW of approximately 11000. This was in agreement with MWs determined by sedimentation equilibrium studies in the analytical ultracentrifuge. Experimental plots of r^2 (r is distance from the axis of rotation) versus log c (log of concentration of the protein, expressed as fringe displacements) for the meniscus depletion experiment, and of $(1/r)(\partial c/\partial r)$ versus c for the low-speed method were linear confirming homogeneity of the ferredoxin and giving calculated MWs of 11800 and 12500, respectively. These may be slightly high since the partial specific volume of 0.715 used in calculations was derived from amino acid composition, and does not take into account the influence of the [2Fe–2S] cluster; this might be expected to decrease the partial specific volume [21]. Clearly though the MW of *Rhodymenia palmata* ferredoxin is the same as that of nearly all other cyanobacterial and algal ferredoxins [16].

Biochemical properties

The biological activity of *Rhodymenia palmata* ferredoxin was assessed in two assay systems. In catalysis of $NADP^+$ photoreduction by barley chloroplasts depleted of native ferredoxin the algal ferredoxin supported a rate of 45 μmol of $NADP^+$ reduced per hr per mg of chlorophyll, the same as that by *Porphyra umbilicalis* ferredoxin in parallel assays. No pyridine nucleotide photoreduction was observed if ferredoxin was omitted, or if saturating levels of ferredoxin were present but chloroplasts omitted.

The activity of *Rhodymenia palmata* ferredoxin was also measured by its ability to support pyruvate decarboxylation by the phosphoroclastic system of *Clostridium pasteurianum* depleted of native low potential electron acceptors. This has proved a useful system for assessing electron transferring ability of ferredoxins or flavodoxins though pyruvate decarboxylation by this system probably does not occur in red or green algae, though it does in some cyanobacteria [22]. In this system the activity of *Rhodymenia palmata* ferredoxin was slightly less than that of *Porphyra umbilicalis* ferredoxin, but nevertheless in the same range as activities of cyanobacterial and plant ferredoxins [16]. The Km_{Fd} of the red algal ferredoxins in this system was *ca* 4×10^{-5} M, ten-fold higher than that for the native ferredoxin.

Amino acid sequence

The *N*-terminal sequence of *Rhodymenia palmata* ferredoxin was determined on a sequencer by using *ca*

Table 2. *N*-Terminal amino acid sequences of algal ferredoxins

	1				5				10				15				20				25	
Rhodymenia palmata	Ala-	Val-	Lys-	Tyr-	Thr-	Val-	Thr-	Leu-	Ser-	Thr-	-	-	Pro-	Gly-	Gly-	-	-	Val-	Glu-	Glu-	Ile-	Glu-	Gly-	Asp-	Glu-	Thr-	Ser-
Porphyra umbilicalis	Ala-	Asp-	-	-	Tyr-	Lys-	Ile-	His-	Leu-	Val-	Ser-	Lys-	Glu-	Glu-	Gly-	Ile-	Asp-	Val-	Thr-	Phe-	Asp-	Cys-	Ser-	Glu-	Asp-	Thr-	
Cyanidium caldarium	Ala-	Ser-	-	-	Tyr-	Lys-	Ile-	His-	Leu-	Val-	Asn-	Lys-	Asp-	Gln-	Gly-	Ile-	Asp-	Glu-	Thr-	Ile-	Glu-	Cys-	Pro-	Asp-	Asp-	Gln-	
Scenedesmus quadricauda	Ala-	Thr-	-	-	Tyr-	Lys-	Val-	Thr-	Leu-	Lys-	Thr-	-	-	Pro-	Ser-	Gly-	-	-	Asp-	Gln-	Thr-	Ile-	Glu-	Cys-	Pro-	Asp-	Asp-	Thr-
Dunaliella salina I	-	-	Ser-	-	-	Tyr-	Met-	Val-	Thr-	Leu-	Lys-	Thr-	-	-	Pro-	Ser-	Gly-	-	-	Glu-	Glu-	Lys-	Val-	Glu-	Val-	Ser-	Pro-	Asp-	Ser-
Dunaliella salina II	Ala-	-	-	-	-	Tyr-	Lys-	Val-	Thr-	Leu-	Lys-	Thr-	-	-	Pro-	Ser-	Gly-	-	-	Asp-	Gln-	Thr-	Ile-	Glu-	Val-	Ser-	Pro-	Asp-	Ala-

	26				30				35				40				45						50
Rhodymenia palmata	Tyr-	Val-	Leu-	Asp-	Ser-	Ala-	Glu-	Asp-	Gln-	Gly-	Ile-	Asp-	Leu-	Pro-	Tyr-	Ser-	(Cys)-	(Arg)-	Ala-	Gly-	Ala-	X -	(Ser)-	Asp-	(Cys)-
Porphyra umbilicalis	Tyr-	Ile-	Leu-	Asp-	Ala-	Ala-	Glu-	Glu-	Glu-	Gly-	Ile-	Glu-	Leu-	Pro-	Tyr-	Ser-	Cys -	Arg -	Ala-	Gly-	Ala-	Cys-	Ser -	Thr-	Cys -
Cyanidium caldarium	Tyr-	Ile-	Leu-	Asp-	Ala-	Ala-	Glu-	Glu-	Gln-	Gly-	Leu-	Asp-	Leu-	Pro-	Tyr-	Ser-	Cys -	Arg -	Ala-	Gly-	Ala-	Cys-	Ser -	Thr-	Cys -
Scenedesmus quadricauda	Tyr-	Ile-	Leu-	Asp-	Ala-	Ala-	Glu-	Glu-	Ala-	Gly-	Leu-	Asp-	Leu-	Pro-	Tyr-	Ser-	Cys -	Arg -	Ala-	Gly-	Ala-	Cys-	Ser -	Ser-	Cys -
Dunaliella salina I	Tyr-	Ile-	Leu-	Asp-	Ala-	Ala-	Glu-	Glu-	Ala-	Gly-	Val-	Asp-	Leu-	Pro-	Tyr-	Ser-	Cys -	Arg -	Ala-	Gly-	Ser-	Cys-	Ser -	Ser-	Cys -
Dunaliella salina II	Tyr-	Ile-	Leu-	Asp-	Ala-	Ala-	Glu-	Glu-	Ala-	Gly-	Leu-	Asp-	Leu-	Pro-	Tyr-	Ser-	Cys -	Arg -	Ala-	Gly-	Ala-	Cys-	Ser -	Ser-	Cys -

The sequences are taken from [2], except *Porphyra umbilicalis* [9] and *Rhodymenia palmata* (present work), and were aligned to give maximum similarity. Residues in brackets are less certain.

500 nmol of carboxymethylated protein. Unambiguous identification of most residues was possible, although after 40 residues buildup of background prevented unequivocal identifications in some cases. The partial sequence is compared (Table 2) with the corresponding data for five other algal ferredoxins so far studied, the sequences being arranged for maximum similarity. Compared with the other algal ferredoxins, and with those from other sources so far reported, *Rhodymenia palmata* ferredoxin has an additional residue at position 3. Though an insertion at this position also occurs in ferredoxin I from *Nostoc* strain MAC in this latter case it is one of the two additional residues invariably present when proline at position 12 is absent [16]. *Rhodymenia palmata* ferredoxin possesses proline at this position, though it is absent in the proteins from *Porphyra umbilicalis* and *Cyanidium caldarium*.

Rhodymenia palmata ferredoxin shows more similarity to the green algal ferredoxins than to those from *Porphyra umbilicalis* and *Cyanidium caldarium* (Table 3). Of this small group of algal ferredoxins most similarity occurs between the ferredoxins of the green algae, and between *Cyanidium* ferredoxin and those of *Porphyra* and *Scenedesmus*. Possible relationship of *Porphyra* and *Cyanidium* on this basis have been emphasised elsewhere [2]. On the basis of the first forty residues *Rhodymenia palmata* ferredoxin shows at least 17 differences compared to any other [2Fe–2S] ferredoxin for which sequence data are published. Within this group it is the only ferredoxin to possess valine at positions 2 and 16, threonine at 5 and 24, serine at 9, 25 and 30, and glycine at 13 and 21; the latter is noteworthy since all other [2Fe–2S] ferredoxins so far sequenced possess either valine or cysteine at position 21. These features appear to have conferred little change in the properties of *Rhodymenia* ferredoxin compared to, for example, *Porphyra umbilicalis* ferredoxin, though the former is unusual in its tendency to aggregate readily. The diversity from *Cyanidium caldarium* ferredoxin is somewhat surprising; in immunodiffusion studies antibody to phosphorylase isoenzyme a_2 from *Cyanidium caldarium* reacted best with isoenzymes isolated from *Rhodymenia pertusa* and two cyanobacteria, but less well with isoenzymes from two green algae [23].

On the basis of this limited information *Rhodymenia palmata* ferredoxin appears to be as distinct from the other algal ferredoxins as from ferredoxins from other sources. It is of interest that the cytochrome *c* sequences of *Rhodymenia palmata* and *Porphyra umbilicalis* also showed considerable differences [24].

Table 3. Matrix of amino acid differences in first 50 positions of algal ferredoxins. I and II refer to two different ferredoxin types

		(a)	(b)	(c)	(d)	(e)	(f)
Rhodymenia palmata	(a)						
Porphyra umbilicalis	(b)	26					
Cyanidium caldarium	(c)	23	13				
Scenedesmus quadricauda	(d)	19	18	13			
Dunaliella salina I	(e)	20	24	21	13		
Dunaliella salina II	(f)	19	19	16	5	10	

EXPERIMENTAL

Rhodymenia palmata was harvested locally from the Laminaria zone of the seashore at Aberystwyth (O.S. 579819) during the late summer. The fronds were thoroughly washed, any obviously infected with epiphytic Bryozoa were rejected, and the remainder stored at −20° until required.

Fronds (6 kg wet wt) were freeze-dried (*ca* 1 week) in a commercial freeze-drier (Edwards High Vacuum, Model 30P2/673), and were then milled and sieved through a 1 mm mesh in a Laboratory Mill (Christy & Norris, Chelmsford). The resulting powder was suspended in 6 l. of 0.15 M Tris–HCl, and stirred overnight in a cold room before straining through cheesecloth. Unless stated otherwise buffers were at pH 7.4 at 4°, and all stages of purification were carried out at this temp. DEAE-cellulose (100 g) was suspended in the filtrate after this had been diluted with deionised water (*ca* 10 l.) to conductivity values less than 5 mMho. After stirring for 15 hr the DEAE-cellulose was centrifuged down at 1500 × *g* for 5 min, and washed twice with 300 ml of 0.15 M Tris-HCl before the ferredoxin was recovered in 0.3 M Tris-HCl, 0.55 M NaCl (Cl^- = 0.8 M).

Successive stages in purification involved treatment of the supernatant with deoxyribonuclease (2 mg) and ribonuclease (2 mg) for 30 min at 20° to degrade polynucleotides, and the removal of protein by precipitation with $(NH_4)_2SO_4$ (60% saturation).

The supernatant was then dialysed against 0.15 M Tris-HCl for 12 hr before being loaded on to a DEAE-cellulose column (35 × 5 cm). The column was washed with 1 l. of 0.15 M Tris-HCl containing 0.1 M NaCl (Cl^- = 0.22 M) and then developed with 0.15 M Tris-HCl containing 0.2 M NaCl (Cl^- = 0.32 M) at a flow rate of 60 ml hr^{-1}; fractions (10 ml) containing the reddish-brown ferredoxin ($A_{420nm}/A_{276nm} > 0.1$) were collected. After appropriate dilution the ferredoxin solution was concentrated by passage through a short (6 × 1 cm) DEAE-cellulose column, being eluted in 0.15 M Tris-HCl containing 0.55 M NaCl. It was then subjected to gel filtration on a column (26 × 5 cm) of Sephadex G-50 developed with 0.15 M Tris-HCl containing 0.2 M NaCl at a flow rate of 40 ml hr^{-1}; fractions (5 ml) with $A_{420}/A_{276} > 0.25$ were bulked.

In contrast to similarly purified preparations of ferredoxin from *Porphyra umbilicalis* [8] the ferredoxin from *Rhodymenia palmata* still contained polynucleotide material, as shown by the appreciable absorbance at 260 nm and as a consequence the A_{423}/A_{276} ratio was low; polyacrylamide gel electrophoresis indicated the presence of other minor protein impurities. The polynucleotide material could be removed by gel filtration on a column (72 × 2.5 cm) of Sephadex G-100, but removal of the minor protein contaminants necessitated preparative electrophoresis on a column (13 × 2.5 cm) of 15% polyacrylamide gel (average pore radius 1.22 nm). Electrophoresis of 20 mg amounts of ferredoxin was continued until the visible ferredoxin band was *ca* 1 cm from the bottom of the gel. This zone was excised and the gel shredded and gently shaken for several hours with 10 ml of 0.15 M Tris-HCl in which was suspended 2 g DEAE-cellulose. The suspension was passed through a sintered glass funnel and the filtrate bulked with a further washing with 10 ml 0.3 M Tris-HCl containing 0.55 M NaCl. The ferredoxin from four such experiments was bulked and concentrated on a short DEAE-cellulose column. Analytical polyacrylamide gel electrophoresis showed the presence of small amounts of a second protein but subsequent studies confirmed this was an aggregated form,

possibly a dimer, of the ferredoxin. The final yield of purified ferredoxin was *ca* 50 mg.

Other methods are described elsewhere [16].

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