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CYTOCHROME *cs* FROM *RHODYMENIA PALMATA* AND *PORPHYRA UMBILICALIS* AND THE AMINO ACID SEQUENCES OF THEIR *N*-TERMINAL REGIONS

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Key Word Index—*Rhodymenia palmata*; *Porphyra umbilicalis*; Rhodophyta; basic cytochrome *c*; *N*-terminal amino acid sequence; automatic sequencer; cytochrome purification.

Abstract—Basic *c*-type cytochromes homologous with plant and animal mitochondrial cytochrome *c* have been isolated and purified from *Rhodymenia palmata* and *Porphyra umbilicalis*. The *N*-terminal regions have been analysed using a Beckman 890C automatic sequencer. When compared to animal cytochrome *c*, the *Rhodymenia* cytochrome *c* has an unblocked *N*-terminal tail of 10 amino acids, whereas *Porphyra* has an unblocked *N*-terminal tail of only a single amino acid.

INTRODUCTION

Since Keilin's early work [1, 2] which demonstrated that cytochromes occurred in plants as well as in animals, fungi and microorganisms, cytochrome *c* has been isolated and purified from a variety of higher plants [3, 4]. Very few studies have been done however, on cytochrome *c* from lower plant groups.

In 1935 Yakushiji [5] solubilized a *c*-type cytochrome from a red alga, *Porphyra* sp. Since then several workers have isolated and examined *c*-type cytochromes from a variety of red, brown, green and other algae [6–8]. However, the majority of these *c*-type algal cytochromes have their α-band absorption maximum at 552·5–554·5 nm, and their γ-band absorption maximum at 415–418 nm [6]. They are acidic proteins with high redox potentials of +340–385 mV and are not appreciably oxidized by mammalian cytochrome oxidase [9, 10]. Hence, it is likely that these cytochromes do not participate in cell respiration, but are involved in photosynthesis and are related to cytochrome *f* [10]. Cytochromes homologous with the eukaryotic mitochondrial cytochrome *c*, i.e. basic proteins with an α-band absorption at 550 nm which are active in mammalian oxidase assay systems, have rarely been characterised from algae. Holton and Myers [11, 12] for example, demonstrated the presence of several *c*-type cytochromes in the blue–green alga, *Anacystis nidulans*. One of these was a basic *c*-type cytochrome with an α-band maximum at 552 nm; it was obtained however, only in very low yield. It is possible that the cytochromes c_{549} from *Scenedesmus* [13] *and* c_{550} from *Ochromonas* (Powls, R., personal communication), are also of mitochondrial orgin, since they are basic proteins. Although cytochrome c_{550} from a *Chlamydomonas* mutant is acidic, it reacted rapidly with a mitochondrial oxidase system [14] and so is of uncertain relationship. Meatyard and Boulter [15] have studied the basic cytochrome c_{550} from the green alga, *Enteromorpha intestinalis*. They have determined its amino acid sequence, which clearly demonstrated its homology with mitochondrial cytochromes *c*. Cytochrome c_{558} from *Euglena* has also been studied and shown to be a basic protein more closely associated with aerobic metabolic processes than with the photosynthetic pathways of electron transfer [16, 17]. Sequence studies on this protein [18] confirm its homology with the mitochondrial cytochromes *c*

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Table 1. The purification of *Rhodymenia* cytochrome *c*

Step	Yield (mg/10 kg fr. wt algae*) A	B	Purity $E_{410}(ox)/E_{280}(ox)$
Amberlite CG-50	5·0	16·0	1·2–1·5
CM–Sephadex	5·0	15·0	1·6–1·8
$(NH_4)_2SO_4$ fractionation + Biogel P–30	4·0	13·0	3·0–4·0
CM–52 cellulose	3·2	10·0	4·5–5·0
Desalting (G25 Sephadex)	2·4	8·0	5·0

* Approximately 1·4 kg dry weight.
A—Thalli collected in May; B—Thalli collected in September.

and show that the unusual α-band absorption maximum is due to an abnormal haem linkage.

The present paper reports on the isolation of a basic *c*-type cytochrome from two red algae, which is probably homologous with the mitochondrial cytochrome *c* from higher plants, fungi and animals.

RESULTS AND DISCUSSION

The method used for the purification of the cytochromes was a combination of methods previously used with higher plant materials [3, 4]. The yields and purities at the various stages in the purification are given in Table 1 for *Rhodymenia* and Table 2 for *Porphyra*.

The major problems encountered in the purification were as a result of the low concentration of cytochrome *c* present in the red algae, as compared with animal, fungi and germinating seed sources [3, 4]; to compensate for low yields, a large mass of starting material was required. In order to keep losses in yield to a minimum, speedy handling in the initial extraction stages was required [19], and the use of a Broadbent Centrifuge proved particularly helpful in this respect, as it facilitated the relatively quick bulk filtration of homogenates. No serious problems due to the presence of slime were encountered at this stage in the preparation, and it was found unnecessary, therefore, to use the various methods which had been employed by Sugimura *et al.* [6] to minimise slime production when preparing acidic (photosynthetic) cytochromes *c* from algae. The large volumes and low cytochrome *c* content of filtrates provided major problems of concentration in the initial stages. Cation-exchangers were used and found to be an efficient method of concentrating dilute cytochrome solution. Batchwise elution of the resin after it had been washed by decantation, was shown to be the best method, since much extraneous material precipitated in, and on, the resin. Batchwise elution however, still gave fairly large volumes of eluate which necessitated a further concentration step and CM–Sephadex proved the most useful way of effecting this. For both cytochromes the

Table 2. The purification of *Porphyra*† cytochrome *c*

Step	Yield (mg/10 kg fr. wt algae*)	Purity $E_{410}(ox)/E_{280}(ox)$
Amberlite CG-50	4·5	0·1
CM–Sephadex	4·0	0·2–0·7
$(NH_4)_2SO_4$ fractionation + Biogel P-30	2·6–3·3	1·0–2·5
CM–52 cellulose	1·1	5·8
Desalting (G25 Sephadex)	1·0	5·8

* Approximately 1·5 kg dry weight.
† Thalli collected in May.

Table 3. Comparison of the spectral ratios of cytochromes *c* from red algae and certain animals and higher plants

Species	$\frac{550\ nm(R)}{280\ nm(O)}$	$\frac{416\ nm(R)}{410\ nm(O)}$	$\frac{410\ nm(O)}{280\ nm(O)}$	$\frac{416\ nm(R)}{550\ nm(R)}$
Porphyra umbilicalis	0·9	1·22	5·8	5·3
Rhodymenia palmata	1·0	1·12	5·0	5·3
Horse heart [31]	1·20	1·20	4·6	4·4
Carp [24]	1·06	*	*	*
Wheat [32]	1·16	1·20	3·8	4·4
Castor [3]	1·22	1·25	4·9	5·5

* These ratios were not given by the authors but the spectrum was stated to be similar to other cytochromes *c*. An iron content of 0·44% showed the preparation to be pure. *R*—Reduced form (Fe^{2+}); *O*—oxidized form (Fe^{3+}).

CM–Sephadex step removed large amounts of a yellow-coloured impurity which preceded the cytochrome band during elution.

After initial concentration the cytochrome was brought to a high degree of purity by ammonium sulphate precipitation and gel-filtration through Biogel P-30. Final purification of the cytochrome was by gradient elution from CM–cellulose. This later technique would separate any deamidated forms if they were present. Inclusion of this step was considered necessary, in view of the use of an ammonium sulphate precipitation step, which, it has been claimed, leads to deamidation [20]. However, in this case, there was no evidence that any appreciable deamidation occurred during purification. Dialysis was avoided whenever possible, especially in the later stages, as at low salt concentrations higher plant cytochrome *c* has been reported to be unstable [21], and also shows a tendency to bind to dialysis tubing.

The preparations of *Rhodymenia* and *Porphyra* cytochromes *c* were shown to be greater than 95% pure when examined by acrylamide gel electrophoresis at pH 4·5 and 8·3. The maximum yields obtained for cytochrome from *Rhodymenia* and *Porphyra* were 8·0 and 1·0 mg/kg starting material respectively. By comparison, the yield of crystals of acidic *c*-type cytochrome from *Porphyra* was 13 mg/kg starting material [22]. In the case of *Rhodymenia* it was shown that the yield obtained was very variable with the time of year that the thalli were collected; thalli which were collected in the autumn gave considerably higher yields than those collected in the spring.

Table 3 gives the ratio of the absorbances and spectral characteristics of the oxidised and reduced forms of the purified cytochromes *c*, and also, for comparative purposes, those of horse-heart, carp, wheat and castor cytochromes *c*. Both the red algal cytochromes *c* had α-band absorption maxima at

Table 4. *N*-Terminal regions of *Rhodymenia* and *Porphyra* cytochromes *c* compared with those from other cytochromes *c* of known sequences

	1 5 10 15 20 25
Rhodymenia	NH_2-Ala-Pro-Ala-Ala-Ala-Tyr-Ala-Asp-Leu-Lys-GLY-Asn-Pro-Thr-Lys-GLY-Ala-Lys-Ile-PHE-Lys- ? - ? -(Cys)- ? - ? -(Cys)- ? -
Porphyra	NH_2-Ala-GLY-Asn-Glu-Tyr-Lys-GLY-Ala-Lys-Ile-PHE-Lys- ? - ? -(Cys)- ? - ? -(Cys)- ? -
Enteromorpha [19]	Acetyl-Ser-Thr-Phe-Ala-Asx-Ala-Pro-Pro-GLY-Asx-Pro-Ala-Lys-GLY-Ala-Lys-Ile-PHE-Lys-Ala-Lys-Cys-Ala-Glx-CYS-HIS-
Ginkgo [33]	Acetyl-Ala-Thr-Phe-Ser-Glu-Ala-Pro-Pro-GLY-Asp-Pro-Lys-Ala-GLY-Glu-Lys-Ile-PHE-Lys-Thr-Lys-Cys-Ala-Glx-CYS-HIS-
Euglena [18]	Acetyl-GLY-Asp-Ala-Glu-Arg-GLY-Lys-Lys-Leu-PHE-Glu-Ser-Arg-Ala-Ala-Gln-CYS-HIS-
Crithidia [34]	X-Pro-TML-Ala-Arg-Glu-Pro-Leu-Pro-Pro-GLY-Asp-Ala-Ala-Lys-GLY-Glu-Lys-Ile-PHE-Lys-Gly-Arg-Ala-Ala-Gln-CYS-HIS-
Humicola [35]	NH_2-Ala-Lys-Gly-Gly-Ser-Phe-Glu-Pro-GLY-Asp-Ser-Ala-Lys-GLY-Ala-Asn-Leu-PHE-Lys-Thr-Arg-Cys-Ala-Glu-CYS-HIS-
Neurospora [36]	NH_2-Gly-Phe-Ser-Ala-GLY-Asp-Ser-Lys-Lys-GLY-Ala-Asn-Leu-PHE-Lys-Thr-Arg-Cys-Ala-Glu-CYS-HIS-
Ustilago [37]	NH_2-Gly-Phe-Glu-Asp-GLY-Asp-Ala-Lys-Lys-GLY-Ala-Arg-Ile-PHE-Lys-Thr-Arg-Cys-Ala-Gln-CYS-HIS-
Candida [38]	NH_2-Pro-Ala-Pro-$\frac{Tyr}{Phe}$-Glu-$\frac{Lys}{Gln}$-GLY-Ser-Ala-Lys-Lys-GLY-Ala-Thr-Leu-PHE-Lys-Thr-Arg-Cys-Ala-Gln-CYS-HIS-
Debaryomyces [39]	NH_2-Pro-Ala-Pro-Tyr-Glu-Lys-GLY-Ser-Glu-Lys-Lys-GLY-Ala-Asn-Leu-PHE-Lys-Thr-Arg-Cys-Leu-Gln-CYS-HIS-
Saccharomyces-Iso-1 [40]	NH_2-Thr-Glu-Phe-Lys-Ala-GLY-Ser-Ala-Lys-Lys-GLY-Ala-Thr-Leu-PHE-Lys-Thr-Arg-Cys-Leu-Gln-CYS-HIS-
Saccharomyces-Iso-2 [40]	NH_2-Ala-Lys-Glu-Ser-Thr-Gly-Phe-Lys-Pro-GLY-Ser-Ala-Lys-Lys-GLY-Ala-Thr-Leu-PHE-Lys-Thr- ? -Cys-Gln-Gln-CYS- ? -

Numbering is for *Rhodymenia* sequence. Residues definitely common to all sequences shown are given in capitals. Alignment is relative to GLY(11), GLY(16), PHE(20) and CYS(27). X—The *N*-terminal blocking group for *Crithidia* is not determined. Residue 2 of the *Crithidia* sequence is Σ-*N*-trimethyllysine (=TML).

* No CYS residue was observed as such in either position. These residues were placed by the appearance of a pink Butyl Chloride extract during the sequence analysis after degradation of the second cysteine residue [41].

550 nm, similar to other cytochromes *c* of mitochondrial origin. Previously, the acidic *c*-type cytochromes which had been purified from algal sources had shown α-band absorption maxima of 552·5–554·5. The other spectral characteristics of the red algal cytochromes are also similar with those typical of mitochondrial cytochromes *c* from other sources (see Table 3). However, the ratio of absorbance at E_{550} nm (reduced)/E_{280} nm (oxidised) is much lower than found for most other cytochromes *c*. A similar situation was found for tuna [23], carp [24] and bonito [25] cytochromes *c* and was shown from sequence analyses to be due to the presence of two tryptophan residues. Thus, this ratio suggests that both the red algal cytochromes *c* may also contain two tryptophan residues; preliminary sequence results confirm this conclusion for *Rhodymenia* (Meatyard, B. T. and Boulter, D., unpublished).

The amino acid sequences of the *N*-terminal regions of the two red algal cytochromes have been determined, and are given in Table 4, together with the sequences of the *N*-terminal regions of several other cytochromes *c* for comparison. *Rhodymenia* had an unblocked *N*-terminal tail which was 10 amino acids long; the *N*-terminal tail of *Porphyra* was, in contrast, only one residue long but was similar in also being unblocked. The "tail" of *Rhodymenia* is, therefore, longer than any other found in cytochrome *c*. The two *N*-terminal sequences show little similarity to either themselves or to any of the other cytochromes *c*. Notably, they show little similarity to the *N*-terminal region of *Enteromorpha* [15], or to higher plant cytochromes *c*. Another interesting difference with respect to the plant cytochromes is that there is no evidence that either of the red algal cytochromes contain the unusual amino acid ε-*N*-trimethyllysine (Meatyard and Boulter, unpublished), which is found in all green plant cytochromes *c* so far examined.

Further studies on the sequences of these two cytochromes are now in progress and that of *Rhodymenia* is nearing completion. These more extensive results do suggest similarity with higher plant cytochromes. When the partial sequences were added to the existing ancestral amino acid sequence tree [26], the red algae come together and on the green plant line of descent, well removed from those of the protozoa, including *Euglena*. This placement is not in agreement with the suggestion of several workers [27], who consider the red algae very separate from the green algae. However, there are considerable differences between the two red algal sequences, and, therefore, these preliminary results must await confirmation. The present evidence is however, that the red algae are an evolutionary primitive and diverse group.

EXPERIMENTAL

Plant materials. Rhodymenia palmata was collected from Souter Point, Co. Durham; thalli having epiphytic Bryozoans were rejected. *Porphyra umbilicalis* thalli were collected from pure stands of the alga on rocks at St. Abbs Head, Berwickshire.

Chemicals. Chemicals and reagents were of the highest purity commercially available.

Extraction and purification of cytochrome c. Washed thalli were extracted in batches of 50–70 kg fr.wt. Each batch was blended in *ca* 2 kg quantities in a Waring blender for 3 min with 1 litre of pre-chilled dis. H_2O (2–4°), 20 g ascorbic acid and 1 g EDTA. The pH of the homogenate (60–100 l.) was adjusted to pH 8·0 with 1 M Tris and filtered at 2–4° through Terylene bags (Type 1481, Samuel Hill Ltd., Rochdale, Lancs.) in a 52 cm perforated basket centrifuge (Type 86, Thos. Broadbent, Huddersfield). Concentration of the basic cytochrome from the filtrate by chromatography on Amberlite CG-50 (NH_4^+ form) and further concentration on CM-50 Sephadex were both as described previously [3]. The cytochrome *c* preparation was then subjected to ammonium sulphate precipitation [4]. The cytochrome *c* was kept reduced by the addition of traces of ascorbic acid and the solution maintained at pH 8 with very dil. NH_3 soln. Solid ammonium sulphate was added slowly to give *ca* 10% increases in saturation. Precipitates which formed between additions of ammonium sulphate were removed by centrifugation at 10000 *g* for 10 min. Those precipitates which contained cytochrome *c* were pooled and redissolved in a minimal quantity of dis. H_2O and further purified by chromatography on Biogel P-30, as described in Richardson *et al.* [3]. Final purification of the cytochrome *c* was by gradient elution of the oxidized protein from CM-52 cellulose using a linear gradient of sodium–phosphate buffer, pH 7·2, from 10–300 mM [4].

Polyacrylamide gel electrophoresis, spectrophotometric assay and desalting of purified cytochrome *c*, were as in Richardson *et al.* [3].

Sequence analysis. Automatic sequence analysis was performed on a Beckman 890C sequencer. 3·5 mg samples of *Rhodymenia* cytochrome *c* were analysed using both fast-protein-quadrol and fast peptide programmes. A 1 mg sample of *Porphyra* cytochrome *c* was analysed using the fast-protein-quadrol program. The resulting phenyl–thiohydantoin–amino acids were identified by TLC on silica layers incorporating a fluorescent indicator [28], by GLC on SP-400 (10% v/v) on Chromosorb W and by hydriodic acid regeneration [29, 30].

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