The Amino Acid Sequence of Cytochrome *f* from the Brown Alga *Alaria esculenta* (L.) Grev.

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Cytochrome *f* was isolated from the brown alga *Alaria esculenta* and the amino acid sequence was determined. The native haemoprotein has a molecular weight of 9800 and consists of a single polypeptide chain of 86 amino acid residues with a haem group bonded to cysteine residues at positions 14 and 17. The *N*-terminus is not acetylated and no methylated lysines were found. Sequences of three other algal cytochromes *f* were compared with that of *Alaria* and 22 out of 92 positions were common to the four sequences. One-half of these conserved sites occur between positions 49 and 63. Detailed evidence for the amino acid sequence of *Alaria* cytochrome has been deposited as Supplementary Publication SUP 50048 (6 pages) at the British Library (Lending Division), Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1975) 145, 5.

Cytochrome *f* is an electron carrier in the photosynthetic apparatus of eukaryotic plants and blue-green algae (Kroghmann, 1973). It is a *c*-type cytochrome, but is distinct from respiratory cytochrome *c* in its function and in many of its properties. Cytochrome *f* is membrane-bound in higher plants (Davenport & Hill, 1951) and in some algae (Einor & Mironyuk, 1973). In most algae, however, a soluble easily extracted form occurs which has been isolated from numerous species (Sugimura *et al.*, 1968). The soluble cytochrome is functionally identical with cytochrome *f* of higher plants and its role in photosynthesis has been demonstrated with isolated chloroplasts in *Euglena* (Kato & San Pietro, 1967) and in mutant strains of *Chlamydomonas* (Gorman & Levine, 1965).

A high redox potential (E_0 0.35 V at pH 7), a low isoelectric point (pI 4.0–4.5) and an α -absorption band at 552–554 nm, often with a shoulder near 550 nm, are characteristic properties. The molecule consists of a single polypeptide chain of 80–90 amino acid residues with a haem moiety covalently bonded to the typical sequence Cys-X-Y-Cys-His towards the *N*-terminus.

Pettigrew (1974) compared the amino acid sequences of cytochromes *f* from two unicellular algae, *Euglena gracilis* and *Monochrysis lutheri* and showed 30 positions in common out of 84. A direct comparison of the sequences revealed only traces of homology with cytochrome *c* (Laycock, 1972). General features of the sequence, however, indicate that the tertiary structure of cytochrome *f* may resemble at least part of the cytochrome *c* molecule (Timkovich & Dickerson, 1973).

The comparison of amino acid sequences of homologous proteins has provided a new approach to the study of evolution as well as being a means of examining structural features of the molecule. In general, conserved positions relate to the function of a protein whereas variable regions are considered to reflect its phylogeny. The validity of sequence information, however, depends on the number of sequences and on the diversity of the organisms from which the proteins originate. This report of the amino acid sequence of cytochrome *f* from *Alaria esculenta* is part of a study of the molecular evolution of cytochrome *f* and algal phylogeny. As yet the number of known sequences of cytochromes *f* for comparison are few, therefore any conclusions drawn at this stage must be tentative.

Materials and Methods

Alaria esculenta was collected near Sambro, Nova Scotia, Canada during January to April (1973). Dipeptidyl aminopeptidase was prepared from bovine spleen (McDonald *et al.*, 1972).

Preparation of cytochrome *f*

Whole plants (10 kg) were washed with tap water and soaked for 36 h in 10 mM-NH₃ solution (100 litres) with chloroform (50 ml) at 4°C. The plants were removed, DEAE-cellulose (500 g; Cl⁻ form) was added and the suspension was adjusted to pH 7.0 with HCl. The extract was diluted with distilled water until the electrical conductivity was less than that of a

10mM-KCl solution. The DEAE-cellulose was collected by sedimentation and then washed with distilled water on a Buchner funnel. Cytochrome *f* was eluted by suspending the absorbent in 3 litres of 10%-satd. $(\text{NH}_4)_2\text{SO}_4$ solution which was then adjusted to pH 8.0 with NH_3 solution. The suspension was filtered, adjusted to pH 6.0 with HCl, and solid $(\text{NH}_4)_2\text{SO}_4$ was added to saturate the solution. Precipitated protein was collected by centrifugation then redissolved in water (50ml). The dark-red solution was adjusted to pH 7.0 with NH_3 solution and centrifuged at 20000*g* for 10min. The clear supernatant was then applied to the bottom of a column (5cm×50cm) of Sephadex G-75 equilibrated with 0.02M- NaH_2PO_4 - Na_2HPO_4 , pH 7.0. Fractions containing cytochrome *f* were pooled and diluted with water, if necessary, to give an electrical conductivity less than that of a 10mM-KCl solution. The solution was pumped on to a column (2.5cm×50cm) of DEAE-Sephadex A-25, equilibrated with 0.02M- NaH_2PO_4 - Na_2HPO_4 , pH 7.0, and eluted with a linear concentration gradient up to 0.20M- NaH_2PO_4 - Na_2HPO_4 , pH 7.0, in a total volume of 1 litre. The combined cytochrome-enriched fractions were saturated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitated protein was collected by centrifugation. It was dissolved in a minimum volume of distilled water then dialysed against several changes of distilled water for about 24h. The solution was freeze-dried and stored at -20°C. If the purity of the preparation was not satisfactory, as judged by gel electrophoresis and by the absorption spectrum, it was repurified in the oxidized form on the DEAE-Sephadex column. All procedures were performed at 4°C.

Amino acid-sequence determination

Most of the sequence was determined by analysis of peptides produced by enzymic digestion of the unmodified protein with trypsin and chymotrypsin. Some secondary digests were made with pepsin. Details of the procedures for peptide purification, amino acid analysis and sequence determination by the dansyl-phenyl isothiocyanate method have already been described (Laycock, 1972).

A Beckman automatic sequencer, model 890C, was used for analysis of the *N*-terminal region of the protein. Thiazolinone derivatives were hydrolysed separately with 6M-HCl at 110°C, or with 57% (w/w) HI at 130°C for 22h (Smithies *et al.*, 1971), followed by analysis with a Durrum D-500 amino acid analyser. Asparagine and glutamine phenylthiohydantoins were identified by t.l.c. on silica gel with 15% (v/v) methanol in chloroform.

Dipeptidase digestion

Freeze-dried dipeptidyl aminopeptidase I was dissolved (3mg/ml) in 1% NaCl solution containing

0.5mM-EDTA (disodium salt). The buffer solution (100ml) contained pyridine (0.8ml), 0.1M-HCl (16ml), EDTA (18mg) and 2-mercaptoethanol (0.08ml), and it was adjusted to pH 5.0 with acetic acid. The digestion mixture contained about 100nmol of peptide in water (0.1ml), buffer solution (0.1ml) and enzyme solution (0.1ml), and was incubated at 37°C for 6h. The products were separated by high-voltage paper electrophoresis at pH 6.5. Ninhydrin-positive zones were eluted, acid-hydrolysed and their compositions determined with the amino acid analyser. From the distribution of amino acids on the electrophoretogram the charges on the dipeptides predicted from the sequence showed the positions of amides in the original peptide. A second digest of the peptide, after removal of one residue from the *N*-terminus by the Edman (Hartley, 1970) procedure, was sometimes necessary.

Polyacrylamide-gel electrophoresis

Purified preparations of the cytochrome were analysed for protein contaminants by electrophoresis in 10% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulphate by the method of Weber & Osborn (1969).

Fluorescamine

Peptides were detected on paper chromatograms after marker strips were stained with a 1% solution of fluorescamine in acetone. The strips were dried at room temperature then sprayed with aq. 1% sodium borate, oven-dried and viewed under u.v. light.

Haem cleavage

The haem moiety was removed by the 2-nitrophenylsulphenyl chloride method of Fontana *et al.* (1973). Cytochrome (1–10mg) was dissolved in 90% (v/v) formic acid (0.5ml) with an excess of 2-nitrophenylsulphenyl chloride (10mg). After 10min at 20°C an equal volume of water was added, followed by 3–5ml of ethyl acetate. The apoprotein separated as a white flocculent precipitate, with the haem and excess of 2-nitrophenylsulphenyl chloride remaining in solution. The precipitate was collected by centrifugation and washed twice with ethyl acetate. To remove the nitrophenyl groups from the cysteine residues, the protein was dissolved in 6M-guanidine hydrochloride solution (0.5ml) containing 2-mercaptoethanol (0.1ml) and phenolphthalein indicator. The solution was made alkaline with 10% (w/v) NaOH solution. After 10min at room temperature the reaction mixture was applied to a column (1.5cm×30cm) of Sephadex G-25 (fine) equilibrated

with 50% (v/v) acetic acid. The fractions containing protein were evaporated to dryness on a rotary evaporator.

Peptide nomenclature

Peptides are coded by reference to the digestion procedure, i.e. tryptic (T) or chymotryptic (C), and a number which indicates the position of the peptide in the sequence. Overlapping peptides from the same digest are distinguished by a small letter following the number. Further cleavage is indicated by a second capital letter.

Results

The amount of cytochrome *f* varied with the time of year and very little cytochrome was extracted from plants collected during the summer. Approx. 400mg (40 μ mol) of cytochrome *f* was extracted from 10kg of fresh or frozen whole plants of *Alaria esculenta* which were collected during the winter giving a final yield of 300 mg of purified freeze-dried protein. Some properties of purified cytochrome *f* are listed in Table 1. The high redox potential, the asymmetrical α -absorption band at 553nm, together with a low isoelectric point are characteristic of algal cytochrome *f*. Preparations were considered to be pure when no change in the purity index ($E_{553}/E_{275} = 1.00$) was found after isoelectric focusing in a gradient of pH 3.0–5.0 and when no impurities were detected on polyacrylamide gels.

Amino acid compositions of hydrolysates of the apoprotein (Table 2) show reasonable agreements with the numbers of residues in the proposed sequence. Values for proline were high but there was no evidence of more than two residues in the sequence. Methylated lysine was not detected in the protein, nor in any of the peptides.

Tryptic digests

A colourless precipitate appeared in tryptic digests and this consisted entirely of peptide T4. This insoluble peptide contained the only tyrosine residue in the protein. Chymotrypsin split peptide T4 at tyrosine-49 to give two soluble peptides, a neutral peptide T4Ca, of nine residues, and a basic peptide T4Cb, composed of the remaining six residues, and together the two peptides accounted for the compositions of the insoluble fraction.

The soluble fraction was resolved into nine peptides by high-voltage paper electrophoresis; four basic, one neutral and four acidic peptides. Incomplete hydrolysis at lysine-32 and at lysine-33 resulted in a greater number of peptides than was predictable from the composition of the protein, which showed one arginine and six lysine residues. The neutral peptide T3a with lysine-33 at the *N*-terminus and the basic peptide T2a with lysine-32 and lysine-33 at the *C*-terminus account for the two extra peptides. T2c was lysine-33 and peptide T2b contained the remaining three residues of peptide T2a. The fourth basic peptide consisted of nine residues with arginine at the *C*-terminus.

Four acidic zones were eluted from the pH6.5 electrophoretogram. The haem peptide moved only a short distance from the origin. It was eluted with approximately equimolar quantities of peptide T6. A similar amount of peptide T6 was also recovered from a zone close to the dansylsulphonic acid marker. This large acidic peptide gave a very weak colour with cadmium acetate–ninhydrin but it was readily detectable with fluorescamine. Peptide T6 was split at phenylalanine-76 with chymotrypsin to give an acidic peptide of twelve residues T6Ca and a neutral peptide T6Cb which consisted of the remaining seven residues. Both peptides, T6Ca and T6Cb, were also recovered after chymotryptic digestion of the haem fraction. Peptide T7, an Ehrlich-positive tripeptide with no lysine or arginine and presumably from the

Table 1. Some properties of *Alaria cytochrome f*

The electrode potential was measured at pH7.0 in a $\text{Fe}(\text{CN})_6^{3-}-\text{Fe}(\text{CN})_6^{4-}$ system similar to that of Davenport & Hill (1951). Calculations were based on $E_0' = +0.43\text{ V}$. The molecular weight was determined by sedimentation equilibrium at 2mg/ml in 0.1 M-sodium phosphate buffer at pH7.0. The solution was contained in a double-sector cell and centrifuged at 24620 rev./min for 72 h at 20°C. Data from schlieren patterns were plotted as $\ln(1/x)$ (dc/dx) against x^2 , where x is the distance from the axis of rotation and dc/dx the change in protein concentration with x (Schachman, 1957). The isoelectric point was obtained after isoelectric focusing in an LKB Ampholine column, pH range 3–6 (Laycock & Craigie, 1971). Extinction coefficients were taken from the absorption spectrum of the reduced protein and the concentration was determined by amino acid analysis.

E_0'	Mol.wt.	pI	ϵ_{mM}		
			553 nm	418 nm	280 nm
+0.37 V	10000 $\pm$ 500	4.00 (reduced)	24.5	269.4	21.3

Fig. 1. Amino acid sequence of *Alaria cytochrome*

Peptides are shown above and in relation to their positions in the complete sequence. Solid lines indicate quantitative analysis. — denotes sequence analysis by the dansyl-phenyl isothiocyanate method and - - - indicates a substandard identification by this method. Amino acids released with carboxypeptidase A are shown by —. Dipeptides released by digestion with dipeptidyl aminopeptidase I are shown by —. Quantitative analyses of peptide C3a and T4 were substandard; 0.3 Ser = 1 in C3a and 1.7 Ser = 1 in T4. Both involve serine-43 (see the text).

Table 2. *Amino acid composition of Alaria cytochrome f*

The results from the amino acid analyser were processed by the least-squares method of Katz (1968) to give the values in the Table, which are expressed as residues per molecule. Apoprotein (50–100 nmol) was hydrolysed in evacuated sealed tubes in 6M-HCl at 110°C for 24, 48 and 72 h, and analyses were performed with a JEOL 5AH amino acid analyser. The short column was operated at 28°C with a 0.35M-sodium citrate buffer, pH 6.8 (flow rate 0.62 ml/min) to separate methylated lysines. Cysteine was determined as cysteic acid and tryptophan was estimated in the unmodified protein by the spectrophotometric method of Goodwin & Morton (1946).

Time of hydrolysis (h) ...	Residues/mol				Sequence
	24	48	72	Average or extrapolated	
His	1.1	0.9	0.9	1.0	1
Lys	6.0	6.3	6.0	6.1	6
Arg	1.3	1.2	1.2	1.2	1
Asp	18.2	17.9	17.4	17.8	18 { 11 Asn 7 Asp
Thr	5.7	5.4	5.2	5.2	6
Ser	4.5	4.1	3.9	4.7	4
Glu	6.9	6.7	6.5	6.7	6 { 3 Gln 3 Glu
Pro	2.7	2.6	2.6	2.6	2
Gly	6.9	6.4	6.2	6.5	6
Ala	9.5	9.7	9.5	9.6	10
Val	4.9	5.0	5.0	5.0	6
Met	2.2	2.5	2.3	2.3	3
Ile	5.6	5.7	5.6	5.6	6
Leu	3.9	3.8	3.7	3.8	4
Tyr	0.8	1.1	0.8	0.9	1
Phe	3.0	2.9	2.8	2.9	3
Trp				1	1
Cys				1.3	2

C-terminus of the protein, was well separated from T6 and the remaining acidic peptide T3b on the electrophoretogram.

Chymotryptic digests

Thirteen soluble peptides were isolated from the chymotryptic digests. No insoluble peptides were found. In general, the chymotryptic peptides required more extensive purification than did the tryptic peptides and this resulted in lower recoveries. The haem peptide, however, was recovered in a fairly pure state after high-voltage paper electrophoresis at pH 6.5.

Sequence determination

Evidence for the amino acid sequence is summarized in Fig. 1. Most of the data for the sequence are based on two tryptic and two chymotryptic digests, each of 3 μ mol. The sequence of residues 19–23 was difficult to establish because the manual phenylisothiocyanate degradation would not proceed beyond alanine-16; also there was no evidence of hydrolysis at histidine-18. The sequence beyond the haem-

binding region was established with the aid of an automatic sequence analyser.

Amino acid phenylthiohydantoin were identified unambiguously by a combination of amino acid analysis after hydrolysis in HCl or HI or by t.l.c. up to position 23. The recovery of aspartate phenylthiohydantoin at step 23 was 5% when compared with that of step 1 and further degradation showed only aspartate above the background on amino acid analysis. The yields of amino acid phenylthiohydantoin did not fall sharply after steps involving the haem-binding sites at positions 14 and 17 as noted by Machleidt *et al.* (1973). The average losses were 4% at each step.

The proposed sequence at positions 41–44 depends on the dansyl-phenylisothiocyanate data for peptide T4Ca. Although the identification of these residues was clear, quantitative analyses of peptides C3a and T4 leave some doubt regarding serine-43. Quantitative analysis of peptides overlapping this hydrophobic region would require longer times for complete hydrolysis. Peptides C3 and T4, on the other hand, both show too much serine. This is most probably due to contamination caused by adsorption to the hydrophobic regions of these peptides.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27						
1	Monochrysis lutheri		Gly	Asp	Ile	Ala	Asn	Gly	Glu	Gln	Val	Phe	Thr	Gly	Asn	Cys	Ala	Cys	His	Ser	Val	Glx	Glx	Mml	Thr	Leu	Glu						
2	Euglena gracilis						Gly	Gly	Ala	Asp	Val	Phe	Ala	Asp	Asn	Cys	Ser	Thr	Cys	His	Val	Asn	Gly	Gly	Asn	Val	Ile	Ser	Ala				
3	Porphyra tenera		Ala	Asp	Leu	Asp	Asn	Gly	Glu	Lys	Val	Phe	Ser	Ala	Asn	Cys	Ala	Ala	Cys	His	Ala	Gly	Gly	Asn	Asn	Ala	Ile	Met	Pro				
4	Alaria esculenta		Ile	Asp	Ile	Asn	Asn	Gly	Glu	Asn	Ile	Phe	Thr	Ala	Asn	Cys	Ser	Ala	Cys	His	Ala	Gly	Gly	Asn	Asn	Val	Ile	Met	Pro				
	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	
1	Leu Ser Ser		Leu	Trp	Lys	-	-	Ala	Lys	Ser	Tyr	Leu	Ala	Asn	Phe	Asn	Gly	Asp	Glu	Ser	Ala	Ile	Val	Tyr	Gln	Val	Thr	Asn	Gly	Lys	Asn		
2	Gly Lys Val		Leu	Ser	Lys	Thr	Ala	Ile	Glu	Glu	Tyr	Leu	Asp	Gly	Gly	Tyr	-	Thr	Lys	Glu	Ala	Ile	Glu	Tyr	Gln	Val	Arg	Asn	Gly	Lys	Gly		
3	Asp Lys Thr		Leu	Lys	Lys	-	Asp	Val	-	-	Leu	Glu	Ala	Asn	Ser	Met	Asn	Thr	Ile	Asp	Ala	Ile	Thr	Tyr	Gln	Val	Gln	Asn	Gly	Lys	Asn		
4	Glu Lys Thr		Leu	Lys	Lys	-	Asp	Ala	-	-	Leu	Ala	Asp	Asn	Lys	Met	Val	Ser	Val	Asn	Ala	Ile	Thr	Tyr	Gln	Val	Thr	Asn	Gly	Lys	Asn		
	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
1	Ala Met Pro		Ala	Phe	Gly	Gly	Arg	Leu	Glu	Asp	Asp	Glu	Ile	Ala	Asn	Val	Ala	Ser	Tyr	Val	Leu	Ser	Lys	Ala	Gly								
2	Pro Met Pro		Ala	Trp	Glu	Gly	Val	Leu	Ser	Glu	Asp	Glu	Ile	Val	Ala	Val	Thr	Asp	Tyr	Val	Tyr	Thr	Gln	Ala	Gly	Gly	Ala	Trp	Ala	Asn	Val	Ser	
3	Ala Met Pro		Ala	Phe	Gly	Gly	Arg	Leu	Val	Asp	Glu	Asp	Ile	Glu	Asp	Ala	Ala	Asn	Tyr	Val	Leu	Ser	Gln	Ser	Glu	Lys	Gly	Trp					
4	Ala Met Pro		Ala	Phe	Gly	Ser	Arg	Leu	Ala	Glu	Thr	Asp	Ile	Glu	Asp	Val	Ala	Asn	Phe	Val	Leu	Thr	Gln	Ser	Asp	Lys	Gly	Trp	Asp				

Fig. 2. Comparison of the amino acid sequences of algal cytochromes *f*
For details see the text.

Amide assignments

The sequence shows 24 positions that could be amidated. This is a high proportion of the total number of residues in the protein and additional evidence was required to make all of the amide assignments. Beside peptide mobilities (Offord, 1966), analysis of carboxypeptidase digests and assignments based on the specificity of chymotrypsin or pepsin, electrophoretic mobilities of dipeptides from dipeptidyl aminopeptidase I digests proved to be very useful. Additional evidence for amides near the *N*-terminus of the protein was obtained with an automatic sequence analyser. A single dipeptidyl aminopeptidase I digest of peptide T1Ca was not sufficient to allow all the amides to be assigned. A second digest, after removal of the *N*-terminal residue by phenylisothiocyanate degradation, provided further evidence. Asparagine-5 was deamidated during the degradation and the dipeptide Asn-Asp was recovered after dipeptidyl aminopeptidase I digestion. Results from the automatic sequencer clearly showed asparagine at both positions 4 and 5. The susceptibility of asparagine to deamidation in the sequence Asn-Gly has been reported (Pettigrew, 1974).

Discussion

A sufficient number of overlapping peptides was isolated from tryptic and chymotryptic digests to deduce the complete sequence of the protein. No peptides were found that were inconsistent with the proposed sequence. The molecule consists of a single polypeptide chain of 86 amino acid residues with a haem moiety bonded to cysteines at positions 14 and 17. The *N*-terminus is not acetylated and no methylated lysines were found.

In Fig. 2 the amino acid sequences of three algal cytochromes *f* are compared with that from *Alaria*. These are from *Monochrysis lutheri* (Laycock, 1972), *Euglena gracilis* (Pettigrew, 1974) and *Porphyra tenera* (R. P. Ambler, personal communication). To accommodate the *Euglena* sequence into Fig. 2 some gaps are postulated in the other sequences resulting in different position numbers from those in Fig. 1. The numbering used below relates to Fig. 2. A total of 92 positions are compared, 22 of which are common to the four sequences. The conserved residues are concentrated near the haem-binding regions and particularly leading to methionine-61, the most probable sixth haem ligand-binding site. The equivalent region in cytochrome *c* is also conserved as are glycine-6 and phenylalanine-10. Position 13, however, is occupied by asparagine in the four sequences but it is a basic residue in cytochrome *c*. The occurrence of groups of residues in distal regions of cytochrome *f* such as the acidic group at 70–72 and the hydrophobic group at 79–81 could correspond to similar

<i>Porphyra</i>	27		
<i>Euglena</i>	58	60	
<i>Monochrysis</i>	49	64	60
	<i>Alaria</i>	<i>Porphyra</i>	<i>Euglena</i>

Fig. 3. Difference matrix of alignments shown in Fig. 2

For details see the text.

regions in cytochrome *c* at positions 90 and 93 (acidic) and 96 and 97 (hydrophobic). Thus if cytochrome *f* and cytochrome *c* had a common ancestor the deletions or insertions that have resulted in the different-sized molecules probably occurred in the middle of the sequence, rather than at the ends.

A difference matrix of the alignments shown in Fig. 2 is given in Fig. 3. The closest relationship between any two of the four sequences is between *Alaria* and *Porphyra* with 27 differences out of the 92 positions that were compared. This number contrasts with 46–60 differences between any two other sequences. It is unwise to generalize from so few data, but it is clear that the four sequences are homologous and that the numbers of differences are in a range that makes cytochrome *f* a suitable molecule for a study of phylogenetic relationships within the algae.

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