The Amino Acid Sequence of Plastocyanin from *Chlorella fusca*

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The amino acid sequence of the plastocyanin from the green alga *Chlorella fusca* was determined. The protein consists of a single polypeptide chain of 98 residues, and was determined by characterization of chymotryptic and thermolysin peptides. The amino acid sequence shows considerable similarity to that of higher plant plastocyanins. The protein contains a single cysteine, and the sequence in the vicinity of this residue is similar to that around the cysteine residue of bacterial azurins. The plastocyanin contains some uncharacterized carbohydrate. Detailed evidence for the sequence of the protein has been deposited as Supplementary Publication SUP 50 036 (17 pp., 1 microfiche) at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms given in *Biochem. J.* (1973) 131, 5.

Plastocyanin is a blue copper protein of low molecular weight that functions in photosynthetic electron transport in algae and plants. The protein was first found by Katoh (1960) in the green alga *Chlorella ellipsoidea*, and has since been shown to be present in the chloroplasts of higher plants (Katoh *et al.*, 1961), and in the blue-green bacterium *Anabaena variabilis* (Lightbody & Krogmann, 1967). When present it occurs in large amounts, and can be readily extracted. The protein is believed to be an essential part of the electron-transport system, and to link photosystems 1 and 2 (Gorman & Levine, 1966; Arnon *et al.*, 1970).

Plastocyanin does not seem to be present in all blue-green bacteria, and has not been found in any of the other classes of photosynthetic bacteria. It has not yet been reported from red algae, a group which has been considered to be among the most primitive photosynthetic eukaryotes (Klein, 1970). Small copper proteins of generally similar properties (e.g. stellacyanin; Malmström *et al.*, 1970) have been found in some non-photosynthetic plant tissues.

Native oxidized plastocyanin has an intense blue colour, much more intense than that of the same amount of copper associated with denatured protein, amino acids or ammonia. Similarly intensely blue complexes exist as some of the copper-containing oxidases (e.g. laccase, ascorbate oxidase and caeruloplasmin; Malkin & Malmström, 1970), and in the bacterial electron-transport protein azurin (Verhoeven & Takeda, 1956; Ambler & Brown, 1967). Plastocyanin is the simplest of these proteins, having a single copper atom and a polypeptide chain of about 100 residues (Milne & Wells, 1970).

The study of the structures of plastocyanins is attractive for comparative purposes, because of its

widespread distribution in higher plants, algae and blue-green bacteria, its small size, and the ease with which it can be purified in adequate amounts.

The amino acid sequences of the proteins from French bean (*Phaseolus vulgaris*; Milne *et al.*, 1974) and potato (*Solanum tuberosum*; Ramshaw *et al.*, 1974) have been determined, and sequence studies of several other higher plant plastocyanins are in progress (Ramshaw *et al.*, 1973). In the present paper evidence is presented for the amino acid sequence of the plastocyanin from the green alga *Chlorella fusca*. A preliminary account has been published (Kelly & Ambler, 1973).

Experimental

Materials

Organism. Spray-dried cells of *Chlorella fusca* var. *vacuolata* (Krauss strain Tokugawa/Tokyo 27) were a gift from the Department of Applied Algology, Trebon, Czechoslovakia, where they had been grown in bacteria-free medium under artificial illumination.

Enzymes and chromatography materials. These were obtained from the sources listed by Ambler & Wynn (1973).

Methods

Preparation of protein. The method used was essentially the same as that described by Gorman & Levine (1966) for the protein from *Chlamydomonas reinhardtii*. The *Chlorella* plastocyanin behaved very similarly to the *Chlamydomonas* protein on DEAE-cellulose for purification steps 1 and 2. Step 3 (gradient elution

from DEAE-cellulose at pH 5.2) was omitted. Gel filtration (step 4) was in 0.01 M-potassium phosphate, pH 7.0 (with no KCl), and Sephadex G-50 was used sometimes instead of Sephadex G-75. After gel filtration, the plastocyanin was absorbed on to a small column of microgranular DEAE-cellulose (Whatman DE-52), equilibrated with 0.01 M-potassium phosphate, pH 7.0, and eluted with a linear KCl gradient. Blue plastocyanin was eluted with about 0.1 M-KCl, and any reduced plastocyanin present was eluted slightly later (about 0.12 M-KCl). For an average preparation (from 1 kg of dry cells), a column of dimensions 1.5 × 1.5 cm diam. was big enough, about one-third of the column being blue at the start of the gradient (0–0.2 M-KCl, total vol. 300 ml). The plastocyanin was then either dialysed against deionized water and freeze-dried or (for preference) precipitated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation, pH 5.5), dissolved in the minimum volume of water, and desalted by gel filtration through Sephadex G-25 into pH 5.1 ammonium acetate (0.05 M-acetic acid adjusted to pH with 2 M- NH_3 solution). Yields were rather variable, ranging from about 5–50 μmol of protein/kg of dry cells. Yields were particularly poor if there had been delays at intermediate steps in the purification.

Properties of *Chlorella fusca* plastocyanin. The spectral properties of the purified protein were similar to those reported for *C. ellipsoidea* plastocyanin (Kato, 1960), although the best value of the absorption index (E_{278}/E_{597} ; Kato *et al.*, 1962) obtained was 2.0, compared with 1.6 for *C. ellipsoidea* and 0.8 for higher plants (Ramshaw *et al.*, 1973). The greater u.v. absorption of the algal proteins is explained by their possession of tryptophan (Table 1), an amino acid absent from at least the majority of examined higher plant plastocyanins.

The *C. fusca* protein appeared homogeneous when subjected to starch-gel electrophoresis at pH 4.0 (0.02 M-potassium hydrogen phthalate) or at pH 6.5 (0.02 M-sodium cacodylate adjusted with HCl), and by N-terminal analysis by the dansyl method.

Carbohydrate analysis by the method of Devor (1950) suggested the presence of up to 6 mol of hexose equivalent/mol of protein, and small amounts of what could have been amino sugars (up to 2 mol/mol) were observed in some amino acid analyses. There is no evidence as to whether this carbohydrate was specifically attached to the protein, or was an artifact of the preparation method.

Amino acid sequence determination. The amino acid sequence was determined by a study of the peptides produced by enzymic hydrolysis of the plastocyanin with chymotrypsin and with thermolysin. The N-terminal sequence was also investigated by automatic phenyl isothiocyanate degradation, and the C-terminal sequence by the characterization of a small peptide formed by CNBr cleavage of the protein and which did not contain homoserine or its lactone.

Before digestion, the protein was either oxidized with performic acid or (for preference) precipitated at 0°C with 2.5% (w/v) trichloroacetic acid, which left the copper in solution. The protein precipitate was then washed twice with 10 vol. of ice-cold acetone, and then dissolved in 0.1 M- NH_3 solution.

The peptides were fractionated by gel filtration followed by high-voltage paper electrophoresis and paper chromatography, and examined for sequence by the dansyl-phenyl isothiocyanate method. In some cases peptides were further degraded by other proteolytic enzymes. The methods used for sequence determination were as those described previously (Ambler & Wynn, 1973; Ambler & Brown, 1967; Ambler, 1973).

Quantities of material used. The sequence determination was carried out on material derived from a single batch of cells, but the products of three independent isolations. Initial experiments used a chymotryptic digest of 10 μmol of performic acid-oxidized protein and a CNBr cleavage of 2 μmol , and further experiments a thermolysin digest of 3.6 μmol of apo-protein and a chymotryptic digest of 2.5 μmol of apo-protein. Quantities of peptides used for sequence determination experiments were in the ranges given by Ambler & Wynn (1973).

Automatic phenyl isothiocyanate degradation. Portions (200 nmol) of native plastocyanin were degraded in a Beckman Model 890A sequenator, by using a 70 min Quadrol double-cleavage programme (Edman & Begg, 1967). The blue film which could be seen at first became colourless at the first reaction. The phenyl thiohydantoins released were identified by t.l.c. on silica-gel layers (Eastman Kodak, Rochester, N.Y., U.S.A.) containing fluorescent indicator (no. 13181) by using the solvent systems of Edman & Sjoquist (1956), and by g.l.c. on SP-400/Chromosorb W.

Results

The amino acid composition of *C. fusca* plastocyanin is shown in Table 1. The ratios of amounts of the stable amino acids present in low concentrations (lysine, histidine, phenylalanine, leucine and proline) are consistent with the protein having a minimum size of about 100 amino acid residues.

The native protein was examined by automatic phenyl isothiocyanate degradation, and 23 out of the first 36 residues were unequivocally identified (Fig. 1). Two of the remaining residues were tentatively identified, but no phenyl thiohydantoins were detected by either t.l.c. or g.l.c. in the other 11 positions. Conventional sequence determination experiments (Fig. 1) showed these undetected residues to be threonine (4), serine (3), asparagine (2), glutamic acid (1) and lysine (1). The sequenator results were completely consistent with those obtained by the other methods. At

Table 1. Amino acid composition of *Chlorella fusca* plastocyanin

Results are shown as residues per molecule. Analysis values were: (1), the average of 12, 24, 48 and 96 h hydrolyses, except for the following amino acids: serine and threonine values were derived by extrapolation to zero time of hydrolysis, valine and isoleucine by extrapolation to infinite time of hydrolysis, cysteine (as cysteic acid) and methionine (as the sulphone) from a performic acid-oxidized sample hydrolysed for 24 h, and tryptophan by the method of Harrison & Hoffman (1961); (2), from a single independent 24 h hydrolysis of native plastocyanin.

	Analysis		Sequence (Fig. 1)
	(1)	(2)	
Glycine	11.5	11.3	11
Alanine	11.8	11.4	11
Valine	9.2	8.4	9
Leucine	4.1	4.0	4
Isoleucine	3.1	2.6	3
Serine	7.6	6.6	7
Threonine	7.7	7.6	8
Aspartic acid	11.2	11.3	6
Asparagine			5
Glutamic acid	10.7	10.3	8
Glutamine			2
Phenylalanine	5.1	5.0	5
Tyrosine	3.7	4.0	4
Tryptophan	0.5		1
Cysteine	0.9		1
Methionine	1.0	0.8	1
Proline	5.1	5.0	5
Lysine	4.0	4.0	4
Histidine	3.0	3.0	3
Arginine	0.0	0.0	0
Total			98

steps where traces of subsidiary phenyl thiohydantoins were detected, the contaminants were consistent with ragged degradation, with no suggestion of microheterogeneity at any site. No attempts were made to measure yields of phenyl thiohydantoins.

The classical evidence for the amino acid sequence is summarized in Fig. 1. This shows all the peptides that were isolated from each digest, together with peptides formed by secondary digestions. Symbols show how much of the sequence of each peptide was determined by the dansyl-phenyl isothiocyanate method, and peptides that were examined with carboxypeptidase. Cases in which the quantitative amino acid analyses or the results of the dansyl-phenyl isothiocyanate degradations were not considered wholly satisfactory are indicated.

Dansyl-phenyl isothiocyanate degradation results were considered unsatisfactory when more than one dansyl-amino acid was identified as being present in significant amount (subjective visual estimate of more than 20%) at a step of the degradation, or when for

some reason (such as accident or shortage of material) the dansyl-amino acid was not definitively identified. The dansyl-phenyl isothiocyanate degradation results obtained with peptides from the first chymotryptic digest (see the Discussion section) were not recorded in the same way as the later ones, and some impurities might not have been noted. For this reason, all of these results are shown on the Figure as unsatisfactory.

The criteria for satisfactory amino acid analyses are: (1) that no impurity should be present in amount larger than 0.20 mol/mol and (2) that when the relative amounts of amino acids present are calculated on the basis that the average amount is integral, no values should fall outside the limits 0.8–1.2, 1.8–2.2, 2.7–3.3 or 3.7–4.3. Nevertheless, values as low as 0.7 (=1) were considered acceptable for tyrosine, as low yields of this amino acid from pure peptides are often found.

The peptides that did not meet these analytical criteria (marked * in Fig. 1) failed for the reasons discussed below.

(1) Peptide H114a contained 33 residues, and is near the maximum size for satisfactory paper purification. The value of serine (3.4 = 4) was low, and of alanine (5.5 = 5) high. There were no indications of a possible serine/alanine dimorphism in this region from any other experiments. The hydrolysis was for only 24 h, so the recoveries for valine (1.6 = 2) and isoleucine (0.6 = 1) were predictably low, since the peptide contains an -Ile-Val- bond.

(2) Peptides C311b and C311c. The only analysis for peptide C311b, which was isolated in only very poor yield, showed considerable contamination (0.36–0.20) with glycine, alanine, leucine, isoleucine, aspartic acid and tyrosine. From peptide C311c, the recovery of isoleucine (0.5 = 1) was very low, suggesting contamination with peptide C311b.

(3) Peptides C16 and C37c. These were peptides from the first chymotryptic digest, and records show the presence of anomalous amounts of phenylalanine, though the actual analysis traces are not now available. As discussed below, it is now believed that the protein contains tryptophan in the position this 'phenylalanine' was thought to occupy.

(4) Peptide C312b. The recovery of the *N*-terminal amino acid, serine (1.5 = 2), was low. Such low *N*-terminal recoveries have been noted in peptides from other proteins purified in similar ways (Ambler, 1973).

(5) Several peptides isolated in low yields, generally the products of minor proteolytic splits, were not isolated sufficiently pure, and contained moderate amounts (0.2–0.35 mol/mol) of unexpected amino acids, or gave results just beyond the permissible stoichiometry limits (e.g. 1.3 or 2.3). Such peptides were H33, H34a, H25, C34c, C37b, C57, C28/11, C28/12 and H15/7N6. Peptide H34a showed lysine

Fig. 1. Amino acid sequence of *Chlorella fusca* plastocyanin

Peptides derived by thermolysin digestion (H) are shown above the sequence, and by chymotryptic digestion (C) and CNBr cleavage (X) below the sequence. Vertical arrows show peptides formed by further digestion. Peptides formed in best yield are shown nearest the sequence. Full lines indicate quantitative amino acid analyses (substandard if marked *). Broken lines indicate qualitative analyses. Under the sequence, — indicates residues identified after degradation of the protein in a sequenator. Under peptide lines, — indicates end-groups and subsequent residues revealed by phenyl isothiocyanate degradation, and identified by the dansyl method (substandard if shown as —). — indicates final residues of peptides identified as the free amino acid after the removal of the remainder by phenyl isothiocyanate degradation. Peptides marked † were examined by carboxypeptidase A digestion. ¹ or ² indicate chymotryptic peptides that were only identified in the first or only in the second digest (see the text). References to the text are given near regions of interest or of conflicting evidence. In the sequence, no residue is numbered 58 or 60, as the best alignment with higher plant plastocyanins (Ramshaw *et al.*, 1974; Milne *et al.*, 1974) requires that two residues be deleted in the algal sequence in this region.

Fig. 1. (continued)

Fig. 1. (continued)

1.4 (=1), but this was probably due to an analyser defect. The full analytical details are given in the Supplementary Publication.

(6) Two peptides from secondary digests gave unsatisfactory analyses. The neutral peptide H35S3 yielded alanine (0.4), valine (1.0) and aspartic acid (1.4), with *N*-terminal valine, interpreted as being a mixture of Val-Asn and Val-Asn-Asn-Ala. Peptide H216S5 analysed as alanine (1.9), glutamic acid (1.1) and aspartic acid (0.38), though no significant contamination was noted during subsequent sequence experiments. The contaminant was probably a peptide rich in aspartic acid, such as peptide H216S6b.

Supplementary publication

Detailed evidence for the amino acid sequence of the protein has been deposited as Supplementary Publication SUP 50036, with the British Library (Lending Division) for storage on microfiche. This evidence comprises:

(1) Tables showing the properties of all the peptides shown in Fig. 1. The successive steps used in the purification of each peptide are given, and where applicable the values of V/V_0 [elution volume/void volume for gel filtration through Sephadex G-25 in 5% (v/v) formic acid] and electrophoretic mobility on paper at pH 6.5. Absolute percentage yields are given for most peptides from primary digests, and relative yields for peptides from secondary digests. Amino acid analyses are given for all peptides, which show impurities present in amounts greater than 0.1 mol/mol.

(2) Further tables show the individual sequence evidence for each peptide. This consists of the *N*-terminal group analysis results, and the results of dansyl-phenyl isothiocyanate-degradation experiments. Details are given where these results are substandard.

(3) The evidence for the presence or absence of amide groups on each aspartic acid and glutamic acid residue in the protein is given.

(4) Details are given of experiments with carboxypeptidase on the peptides marked † in Fig. 1.

Discussion

The evidence for the proposed amino acid sequence is summarized in Fig. 1. The sequence was largely deduced (Kelly, 1971) from a single large chymotryptic digest of performic acid-oxidized plastocyanin, with overlaps deduced by homology with the sequences of some other algal plastocyanins, unpublished because of doubts about the real nature of the organism(s) they were derived from. The evidence at this stage was not altogether satisfactory, as most of the tyrosine in the protein had been converted into 'tyrosine X' (Thompson, 1954) by performic acid oxidation of Cl^- left with the protein from the final ion-

exchange purification step. The evidence for the tryptophan sequence was also poor, and the sequence of two peptides had not been completely determined. This initial evidence was therefore supplemented by the study of a thermolysin digest of apoplastocyanin, which gave in very satisfactory yield peptides that covered the whole sequence, and established most of the postulated overlaps. Because of conflicting results between these two sets of experiments about the nature of residue 30 (see below), a second chymotryptic digest, of apoplastocyanin, was examined, which confirmed the thermolysin results for residue 30. Unfortunately the peptide yields from this final digest were not satisfactory, and no quantitative yields for it are included in the Supplementary Publication. The trouble was probably due to particularly old and dirty pyridine acetate buffer being in the pH 6.5 electrophoresis tank used for the peptide separation. Three degradation experiments were attempted with an automatic protein sequencer, and the results provided additional evidence for several overlaps, as well as giving the only strong evidence for the overlap between residues 21 and 22.

No tryptic digests of this plastocyanin were attempted, as experiments on other algal plastocyanins of similar lysine content (Kelly, 1971) had shown that this resulted in much of the protein being converted into large peptides that were not easy to fractionate by paper methods. The protein contains only a single methionine residue, and CNBr cleavage resulted in a small C-terminal peptide together with high-molecular-weight material. The higher-plant plastocyanins generally contain a second methionine residue at position 58 (Milne & Wells, 1970; Ramshaw *et al.*, 1973), which allows CNBr cleavage to be used very advantageously in the study of their sequences (Ramshaw *et al.*, 1974; Milne *et al.*, 1974), but this residue is deleted in the algal sequence.

Accuracy of proposed amino acid sequence

The strength of the evidence for the sequence varies between different parts of the proposed structure. The parts where the evidence is thought to be weakest are considered in detail below.

The amino acid composition of the protein determined by the analysis of acid hydrolysates (Table 1) is in reasonable agreement with that from the deduced sequence (Fig. 1). This congruency is evidence that the sequence is just 98 residues long, and that no regions had been overlooked by the non-discovery of the relevant peptides in each digest.

Most overlaps have been well established. The weakest is that between residues 21 and 22, for which the only peptide evidence is the impure peptide C311c (see above). The bonds that are here split by chymotrypsin (Thr-Ile and Ile-Lys) are not of types generally considered to be very susceptible to the

enzyme, but very similar behaviour to chymotrypsin has recently been detected for a -Thr-Ile- sequence in a totally different protein (*Rhodopseudomonas spheroides* cytochrome c_2 ; R. P. Ambler, unpublished work). The sequenator results (Fig. 1) confirm that no extra residues locate between positions 21 and 22, and that the peptide juxtaposition is as shown. Only a single residue provides the overlap between residues 38, 39 and 40, but the proposed sequence agrees completely with expectations of protease specificity, and no combination of the available peptides can be made to fit except the arrangement shown in Fig. 1.

There was a discrepancy between the initial and subsequent experiments over the identity of residue 30. The early results indicated that it was phenylalanine, although the yields of the peptides concerned (C16 and C37c) were poor, and tryptophan was known to be present somewhere in the sequence. The later experiments, with protein in which the tryptophan had not been modified by performic acid, showed clearly that tryptophan was located at residue 30, and that there was no phenylalanine there. The sequenator experiments also unequivocally showed tryptophan at position 30.

Early tentative results suggested a different sequence in the 43–47 region to that proposed in Fig. 1. These results were based on dansyl-phenyl isothiocyanate degradation experiments on peptide C111, and suggested five successive acidic (or amide) amino acid residues. In these early experiments, the subjective estimates of dansyl-amino acid-spot intensities on paper or t.l.c. plates were not recorded in the way that has now been standardized, so a full explanation of the discordancy is not possible. It is likely that the results were due to misinterpretation of a degradation that had gone ragged after four or five steps. The sequence shown in Fig. 1 was deduced from degradation experiments on peptides derived by subtilisin digestion of a large thermolysin peptide, and the results were redundant and completely self-compatible. The assignment of free carboxyl groups to each of the residues 43–46 was made on the basis of the mobility changes (at pH 6.5, relative to aspartic acid = -1) for peptide H216S7 (Asp-Glu-Asp-Glu-Val-Pro-Ser-Gly-Ala) from -0.98 to -0.88 to -0.74 to -0.46 as the *N*-terminal three residues were successively removed. The sequence of the 40–55 region was difficult to determine by the methods that were used in this investigation, but would be easy to study now that an enzyme is available that specifically splits peptide bonds on the *C*-terminal side of glutamic acid residues, the extra-cellular protease from *Staphylococcus aureus* (Houmard & Drapeau, 1972).

Assignment of amide or acidic groups throughout the sequence was generally on the basis of the electrophoretic mobilities at pH 6.5 of small peptides, supplemented where appropriate with information from carboxypeptidase experiments. One peptide (H316a,

residues 6–12, Leu-Gly-Ala-Asx-Ser-Gly-Ala) behaved anomalously. When first isolated it had a mobility at pH 6.5 of -0.72, but elution and storage (in 0.1 M-NH₃ solution at -20°C) resulted in partial decomposition to a peptide of mobility -0.40. Peptide H312a, which had mobility -0.40 and amino acid composition indistinguishable from peptide H316a, was isolated from the initial digest in low yield. The expected mobility (Offord, 1966) for peptides of this composition and net charge -1 is -0.39 and net charge -2 is -0.71. There is no evidence for the cause of this anomaly. The more acidic peptide did not have a very acidic mobility at pH 3.5, which would be expected if there was a phosphate group esterifying the serine hydroxyl, and it reacted normally with ninhydrin and dansyl chloride, and had a normal V/V_0 value through Sephadex G-25 in 5% (v/v) formic acid. The attachment of a labile acidic carbohydrate moiety seems a possible explanation.

The sequence shown in Fig. 1 contains two fewer residues than the higher plant plastocyanins (Ramshaw *et al.*, 1974; Milne *et al.*, 1974) in the vicinity of residue 60. It is conceivable that a dipeptide (such as Val-Leu) could have been located in the intact algal protein between residues 56 and 57 (Fig. 1), and lost, perhaps through low ninhydrin colour yield and high chromatographic R_F value, from both chymotryptic and thermolysin digests. The analysis values for peptide H114a, which bridges this gap, are not altogether satisfactory (see above), but do not suggest any such lacuna.

Two peptides of distinct but very similar composition, H410 and H414, were isolated from the thermolysin digest. Peptide H410, isolated in the high yield of 35%, contained two residues of threonine, whereas the more acidic peptide H414 (2% yield) contained only one residue of threonine, but also one residue of glutamic acid, an amino acid absent from peptide H410. These peptides were very easily separated from the remainder of the digest because of their late elution from columns of Sephadex G-25, due to each containing three aromatic residues. Partial sequence determination of peptide H414 indicated that in it residue 80 was glutamic acid, whereas it was threonine in peptide H410. No evidence for this dimorphism was found in the second chymotryptic digest, but the yields of the relevant principal peptides (C37b and C312a) were so low that the detection of a much weaker variant was unlikely.

Sequence relationship to other proteins

The amino acid sequence around the single cysteine residue in the plastocyanin (residue 85, Fig. 1) shows considerable similarity to the sequence around the only free cysteine residue in bacterial azurins (residue 112; Ambler & Brown, 1967; Ambler, 1971). In both classes of proteins this cysteine is close to the

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