CHROMOPHORE-CONTAINING PEPTIDE SEQUENCES IN C-PHYCOCYANIN FROM *MASTIGOCLADUS LAMINOSUS**

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1. Introduction

The blue protein C-phycocyanin comprises a large proportion of the protein of the blue-green algae and is believed to play a primary role in the absorption of light energy for use in photosynthesis. The blue prosthetic group, phycocyanobilin, is a linear tetrapyrrole covalently attached to the protein. Its structure has been established [1–3] but the nature of the covalent link(s) has been variously reported as an ester [4] or both an ester and thioether [5].

Recently our group reported the isolation and characterisation of C-phycocyanin from the thermophylic blue-green alga, *Mastigocladus laminosus*. The monomer of the protein has a molecular weight of 28 000 daltons and contains two subunits each having covalently bound pigment and molecular weight 14 000 daltons but with dissimilar amino acid compositions and N-terminal residues. As the first stage in the establishment of the total structure of C-phycocyanin we now report the amino acid sequences around the point of attachment of the chromophore in each subunit. An interesting similarity to analogous chromophore-containing sequences in cytochromes is noted.

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2. Materials and methods

2.1. *Purification of C-phycocyanin*

Mastigocladus laminosus cells were cultured in Icelandic hot springs at 55° [6] and C-phycocyanin was extracted and purified from the algal cells as described by Binder et al. [7].

2.2. *Isolation of chromophore-containing peptides*

Due to a rapid loss of the blue colour when chromophore-containing peptides were exposed to light or to pH values above 7, all isolation and degradation work was carried out in the dark and wherever possible at low pH.

Approx. 3 μmole C-phycocyanin were dissolved in 50 ml of 5% (v/v) formic acid and 2.7 mg pepsin were added. Digestion was allowed to proceed for 19 hr at 37° after which the digest mixture was dried by rotary evaporation. The dry digest was dissolved in 25 ml 0.05 M pyridine acetate at pH 3.0 and applied to a 2.5 × 92 cm column of SE-Sephadex equilibrated with the same buffer. The column was then eluted successively by 850 ml 0.05 M pyridine acetate buffer, pH 3.0, by 2000 ml of a gradient from the starting buffer to 0.44 M pyridine acetate, pH 4.0, and lastly by 500 ml of 0.44 M pyridine acetate, pH 4.0 (see fig. 1).

The blue coloured peptides were located in peaks A and B. Peak A contained about 16 basic peptides in addition to the single chromophore-containing peptide. The latter was isolated in approx. 20% final yield by two-dimensional high voltage electrophoresis (pyridine–acetic acid–water, 10:1:89, by vol, pH 6.5) and chromatography (butanol–pyridine–acetic acid–

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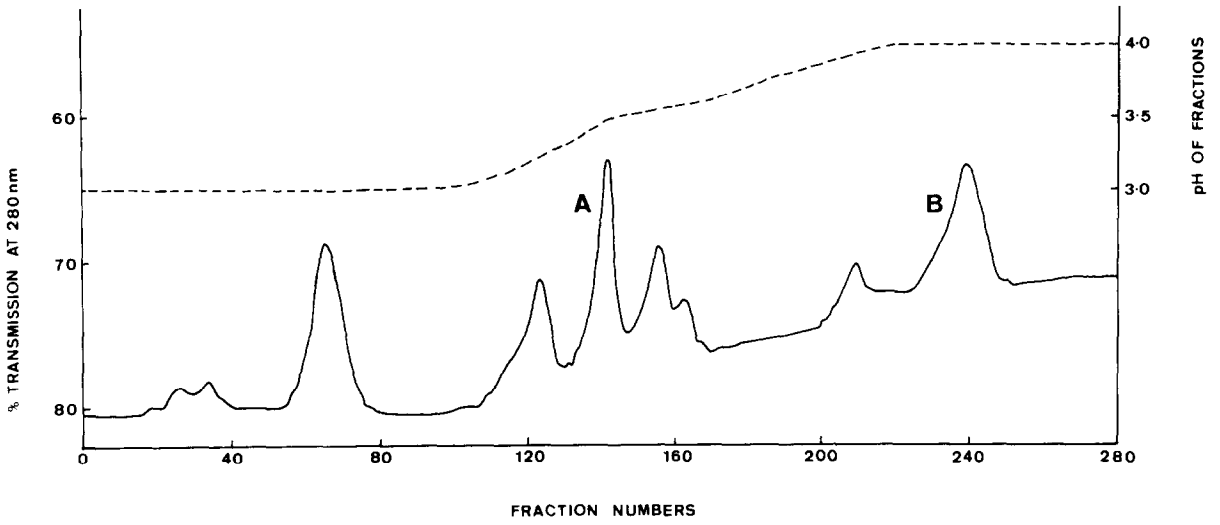

Fig. 1. Chromatography of a peptic digest of C-phycocyanin on a 2.5 × 92 cm column of SE-Sephadex. The sample was applied in 0.05 M pyridine acetate buffer at pH 3.0 and eluted with 850 ml of the same buffer followed by a gradient formed from 1000 ml of this buffer and 1000 ml of 0.44 M pyridine acetate at pH 4.0 and lastly by 500 ml of 0.44 M pyridine acetate pH 4.0. (——) % Transmission at 280 nm; (- - - -) pH of the fractions. The rise in base line in the later part of the chromatogram reflects the increasing pyridine concentration. Peaks A and B contained the blue chromophore-peptides and were pooled for further study.

water, 21:12:2:15, by vol) on Whatman 3 MM paper. Peak B contained a very basic blue peptide in approx. 65% yield and free glycine (approx. 0.7 mole/mole peptide). This sample was not further purified before sequence determination.

2.3. *Tryptic peptides*

Digestions of the isolated peptic peptides with trypsin were performed in 0.1 M ammonium bicarbonate at 37°. Normally enzyme: substrate ratios of between 1:10 and 1:50 were used but to split the Arg–Gly bond between residues 5 and 6 in the peptide from peak B ratios of 1:3 were required. Conversion of the blue colour to weak green or yellow colours occurred during these incubations. The heptapeptide fragment β-T1 was isolated by ion exchange chromatography on a 24 × 0.9 column of SE-Sephadex equilibrated with 0.05 M pyridine acetate at pH 3.0. Elution was achieved with a gradient formed by 60 ml 0.05 M pyridine acetate at pH 3.0 and 50 ml 2.0 M pyridine acetate at pH 5.0. The peptide was eluted at 0.23 M pyridine acetate, pH 3.72. Separation of other tryptic peptides was made by descending chromatography with butanol–pyridine–acetic acid–water, 21:12:2:15, by vol, on Whatman No. 1 paper. Separated peptides were eluted with 10%, v/v, acetic acid.

2.4. *Amino acid analysis*

Peptide samples were normally hydrolysed in 6 M HCl at 110° for 24 hr under nitrogen in sealed tubes. For tryptophan analysis, acid hydrolysis was carried out in the presence of 4% v/v thioglycollic acid [8]. Half-cystine was determined as cysteic acid after performic acid oxidation [9].

2.5. *End-group and sequence determinations*

Amino terminal amino acid determination and dansyl-Edman degradations were carried out as described by Hartley [10]. Dansyl amino acids were identified by chromatography on polyamide sheets using the systems described by Woods and Wang [11], Crowshaw et al. [12] and Hartley [10].

Carboxyl-terminal amino acids were determined by a combination of carboxypeptidase A digestion and dansylation of the liberated amino acids. Carboxypeptidase A was added to the peptide dissolved in 0.1 M $NaHCO_3$. Samples of the digest were then

Table 1
Amino acid compositions of chromophore-containing peptides and their tryptic fragments.

	α-Chain			β-Chain		
	Peptic chromophore peptide	Tryptic subfragments		Peptic chromophore peptide	Tryptic subfragments***	
		α-T1	α-T2		β-T1	β-T2
Asx	5	3	2	2	1	1
Thr	1	1	0	0	0	0
Ser	1	0	1	1	0	0
Glu	0	0	0	0	0	0
Pro	1	1	0	0	0	0
Gly	3	1	2	2	1	1
Ala	3	1	2	3	3	0
Val	0	0	0	0	0	0
$1/2(Cys)_2$*	1	0	1	1	0	0
Met	0	0	0	0	0	0
Ile	3	2	1	1	0	1
Leu	1	0	1	0	0	0
Tyr	0	0	0	1	0	1
Phe	0	0	0	0	0	0
Lys	1	1	0	2	1	0
His	0	0	0	1	0	1
Arg	0	0	0	2	1	0
Trp**	0	0	0	0	0	0
Total	20	10	10	16	7	5
N-terminus	Ile	Ile	Leu	Ala	Ala	Asx
C-terminus	–†	–†	–†	Tyr	–†	Tyr

* Determined as cysteic acid after oxidation with performic acid.
** Determined after hydrolysis in the presence of 4% thioglycollic acid.
*** The two dipeptides seryllysine and cysteinylarginine are omitted.
† Not determined.

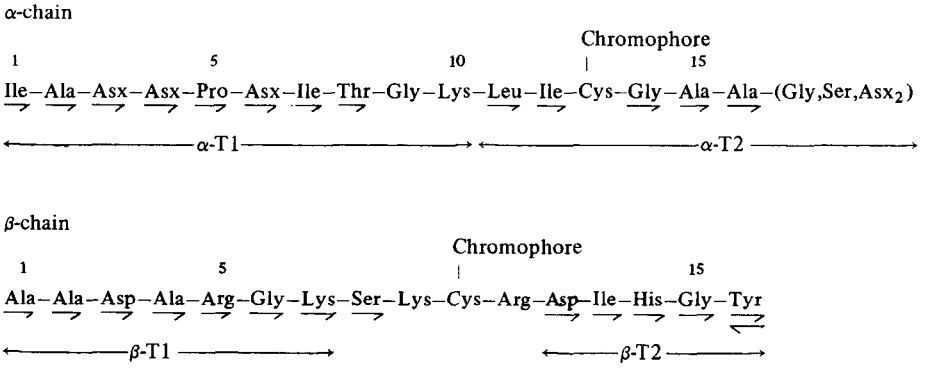

Fig. 2. The amino acid sequences of the two chromophore-peptides isolated from a peptic digest of C-phycocyanin.→, Residues determined by dansylation-Edman degradation;←residue determined by carboxypeptidase A-dansylation.

removed at intervals and added to an equal volume of dansyl chloride solution (2.5 mg/ml in acetone). This procedure terminated the enzyme reaction and labelled the liberated amino acids which were then identified by chromatography on polyamide sheets as above.

2.6. *Cyanoethylation*

Cyanoethylation of peptides with acrylonitrile to block lysine side chain amino groups was carried out by Fletcher's method [13].

2.7. *Free thiol groups*

The presence of free -SH groups was tested with nitroprusside.

3. Results and discussion

3.1. *Assignment of one chromophore-peptide to each subunit chain*

Small amounts of the separated α- and β-subunits of C-phycocyanin, isolated by preparative polyacrylamide gel electrophoresis [7], were digested with pepsin under the same conditions as used for C-phycocyanin. These two digests, a digest of the parent protein and the chromophore-peptides from peaks A and B (fig. 1) were then compared chromatographically on cellulose thin layers with the solvent system butanol–pyridine–acetic acid–water, 21:12:2:15, by volume.

C-phycocyanin gave two blue spots with R_f's 0.31 and 0.57. Peak A and the α-subunit digest each gave one blue spot with identical R_f 0.57. Peak B and the β-subunit digest also gave one blue spot each with identical R_f 0.31. Clearly therefore, the two chromophore peptides came from different subunits of C-phycocyanin. The blue peptide in peak A is from the α-chain and that in peak B is from the β-chain. In addition, the peak B chromophore peptide contains histidine (table 1) which is only found in the β-subunit [7] and confirms its assignment to this subunit.

3.2. *Sequence of the α-chain chromophore peptide*

The amino acid composition of the 20-residue α-chain chromophore peptide is shown in table 1. Cleavage with trypsin at the single lysine residue gave two decapeptide fragments, α-T1 and α-T2, which were separated by paper chromatography; their amino acid compositions are shown in table 1. Peptide α-T2 was pale green. As α-T1 has the same amino terminal residue, isoleucine, as the original peptic peptide and contains the lysine residue it is the amino terminal segment. Peptide α-T2 has amino terminal leucine and no lysine and is therefore C-terminal.

The sequence established by dansylation-Edman degradation of the isolated tryptic peptides is shown in fig. 2. The half-cystine residue at position 13 was not identified directly. Dansylation of the peptide α-T2 after two steps of Edman degradation gave no identifiable dansyl amino acid after hydrolysis but several orange-fluorescing unknown spots. The third Edman degradation of the peptide removed its pale green colour with the N-terminal residue thus proving the chromophore's attachment to residue 13. All amino acids except half-cystine were accounted for in the remaining peptide and it thus occupies position 13 and carries the chromophore. Edman degradation of this peptide fragment could not be taken beyond five steps.

3.3. *Sequence of the β-chain chromophore peptide*

The amino acid composition of the 16-residue chromophore peptide from the β-chain is shown in table 1. It has N-terminal alanine and C-terminal tyrosine. The heptapeptide β-T1 (fig. 2) was isolated from a tryptic digest by ion exchange chromatography on SE-Sephadex and sequenced. As it contains N-terminal alanine and all the alanine residues it is assigned to the N-terminus of the peptic peptide sequence. The Arg–Gly bond between residues 5 and 6 was resistant to normal concentrations of trypsin. Paper chromatography of the tryptic digest showed two spots in addition to the free glycine. At R_f 0.35 was the pentapeptide β-T2 which, as it contains no lysine or arginine but has the C-terminal tyrosine of the peptic peptide, is the C-terminal fragment. The other spot at R_f 0.10 showed, on analysis, the amino acid content of peptide β-T1 plus one residue each of serine and lysine. Dansylation showed two end groups, alanine and serine so that this spot consisted of two peptides, the sequence residues 1–7 and the dipeptide seryllysine. The half-cystine and one residue of the original peptic peptide could not be accounted for in this experiment.

However, when the chromophore-containing peptide was oxidised with performic acid before

tryptic digestion the spot on the chromatogram containing peptide β-T1 and seryllysine had in addition the missing cysteic acid and arginine residues. This indicates the presence of the dipeptide $CysO_3H$–Arg in the digest, or possibly even the tetrapeptide Ser–Lys–$CysO_3H$–Arg since N-terminal cysteic acid was not detected. The bond Lys–$CysO_3H$ might be expected to resist tryptic cleavage due to the strong negative charge on cysteic acid.

Overlap between the N-terminal segment and the two central dipeptides was established by seven dansyl-Edman degradations when serine was found to be residue 8 demonstrating that the heptapeptide β-T1 and seryllysine form the contiguous sequence residues 1–9.

When the peptide was first treated with acrylonitrile to block the ε-amino groups of lysine digestion with high concentrations of trypsin (enzyme/substrate ratio, 1:3, by weight) produced cleavage only at the two arginine residues. Pentapeptide β-T2, already assigned to sequence residues 12–16, was again one of the products and proves that residue 11 is arginine. Residue 10 is therefore half-cystine.

The high concentration of trypsin also gave cleavage at the previously resistant Arg–Gly bond between residues 5 and 6 so that the fragment of sequence residues 1–5 with cyanoethylated amino terminus could be isolated. A small quantity of a peptide with probable composition Gly(1), Ser(1), Arg(1), 1/2$(Cys)_2$(1), N^{ϵ}-(2-cyanoethyl)-Lys(2), consistent with its being the central sequence residues 6–11, was also detected.

Digestion of the peptic peptide with pronase, trypsin and the thermostable aminopeptidase API from *B. stearothermophilus* liberated two residues of aspartic acid so that residues 3 and 12 are both in the acid form. The full sequence is shown in fig. 2.

There was no direct demonstration of the chromophore's attachment to residue 10 in this peptide since the Edman degradation was not carried beyond seven steps.

3.4. *The point of attachment of the chromophore*

C-phycocyanin contains two half-cystines in the α-subunit and one in the β-subunit and there is one free thiol in the α-subunit [14]. But the two remaining half-cystines, although blocked, are not linked as a disulphide bridge since the two chains may be separated by electrophoresis in the presence of urea without pretreatment with disulphide bond breaking reagents [14].

Both chromophore-containing peptides also contained a blocked half-cystine since neither one gives a positive reaction with nitroprusside. In one of these (α) it was clearly established that the chromophore is the blocking group and it is reasonable to suppose that this is also the case in the β-chain.

Inspection of the sequence of the β-chain chromophore peptide reveals an interesting similarity with analogous chromophore-containing sequences in cytochromes. The latter take the general form $\overset{Lys}{Arg}$–Cys–X–X–Cys–His whilst in many plants this may be extended to $\overset{Lys}{Arg}$–X–$\overset{Lys}{Arg}$–Cys–X–X–Cys–His. The partial sequence Lys–Ser–Lys–Cys–Arg–Asp–Ile–His of the β-chain shows a marked resemblance to these general forms with an exchange of isoleucine for one half-cystine.

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