BILIN–APOPROTEIN LINKAGES IN RHODOPHYTAN PHYCOBILIPROTEINS: THE ROLE OF CYSTEINE

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1. Introduction

The photosynthetically active algal biliproteins phycocyanin and allophycocyanin contain the linear tetrapyrrole, phycocyanobilin, which is covalently attached to apoprotein [1]. The structure of phycocyanobilin has been established [2,3] but the nature of the covalent linkages to apoprotein has remained controversial [1]. Attempts to elucidate bilin–apoprotein linkages have included treatment of phycobiliproteins with agents which specifically cleave ester, ether and peptide bonds [4], sequencing [5] and amino acid analysis [6,7] of chromopeptides obtained by proteolysis of phycobiliproteins, and analysis of the pattern of imides derived from the chromophore of phycobiliproteins during chemical degradation in chromic acid [8].

We have shown that phycocyanin and allophycocyanin from the unicellular rhodophyte, *Cyanidium caldarium*, are comprised of dissimilar polypeptide subunits, termed α and β, that the α : β chromophore composition of the phycocyanin subunits is 1 : 2, and that the α : β chromophore composition of the allophycocyanin subunits is 1 : 1 [9,10]. Clearly the complexity of the subunit and chromophore composition of these phycobiliproteins could allow for more than one type of covalent attachment between phycocyanobilin and amino acids in the subunit polypeptides.

O Carra and O hEocha [1] concluded that the best evidence seems to indicate that one type of bilin–apoprotein linkage is a thioether bond between a cysteinyl residue and the ethylidine group of ring I of phycocyanobilin and that a second type of linkage is an ester bond between a seryl residue and the propionic acid carboxyl group in either ring II or ring III of phycocyanobilin (fig.1). We have attempted to modify cysteinyl residues in phycocyanin and allophycocyanin by carboxyamidation to examine the proposed involvement of cysteine in bilin–apoprotein linkages.

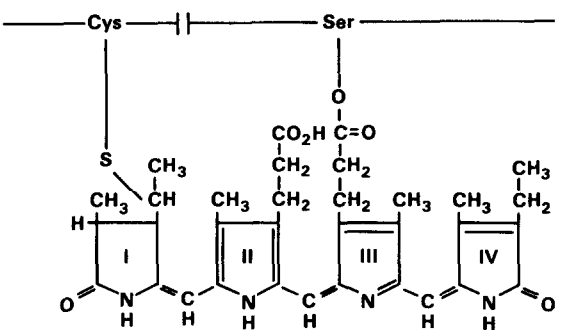

Fig.1. Diagram illustrating proposed linkages between phycocyanobilin and apoprotein [1].

2. Materials and methods

Cyanidium caldarium cells were grown in medium containing inorganic salts as described previously [9] except that 10 mCi of $H_2{}^{35}SO_4$ (New Engl. Nuclear, Boston, MA) was added to the medium and H_2SO_4 was omitted. Radiolabeled phycocyanin and allophycocyanin were isolated from algal cells and purified as described [9,10]. The α and β subunits of phycocyanin were prepared by ion-exchange chromatography on Bio-Rex 70 columns according to the method of Glazer and Fang [11].

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Reduction and alkylation of radiolabeled phycobiliproteins was performed with iodoacetamide (Sigma Chem. Co., St Louis, MO) by a modification of the method of Crestfield et al. [12]. Two mg lyophilized phycobiliprotein were dissolved in 0.5 ml 'carboxyamidation buffer' (8 M urea; 0.36 M Tris–HCl, pH 8.5; 0.05 M dithiothreitol) in a stoppered conical tube. After incubation for 4 h at room temperature in the dark under nitrogen, freshly prepared iodoacetamide in carboxyamidation buffer was added to make the final iodoacetamide concentration 0.1 M. The reaction mixtures were incubated for an additional 30 min at room temperature under nitrogen, excess mercaptoethanol was added to terminate the reaction, and the alkylated phycobiliproteins were dialyzed against distilled water and lyophilized.

Carboxyamidated and non-carboxyamidated phycobiliproteins were hydrolyzed in 6 N HCl at 110°C for 22 h in vacuo. Hydrolysates were analyzed on a Jeolco 6AH automated analyzer employing a 2-column system. A portion of the column eluate was diverted to a fraction collector and an aliquot from each fraction was assayed for radioactivity in 15 ml Scintiverse (Fisher Scientific, Pittsburgh, PA) in a liquid scintillation spectrometer. Amidocarboxymethylcysteine is converted into carboxymethylcysteine (CM-cysteine) during acid hydrolysis.

Crestfield et al. [12] showed that each of the cysteine residues in ribonuclease A was converted into CM-cysteine during incubation with iodoacetic acid. We found that reduction and alkylation of ribonuclease A (Sigma Chem. Co.) with iodoacetamide as described above resulted in complete conversion of cysteine into CM-cysteine.

Carboxyamidated and non carboxyamidated phycocyanin β subunit were degraded in 1% CrO_3–2 N H_2SO_4 at 25°C and 100°C by the procedure of Rudiger and O Carra [8] as modified by Köst et al. [13]. The following authentic imide standards were provided by Dr Hans-Peter Köst, Universität München, München: methylethylidinesuccinimide (MES); hematinic acid (HA); and methylethylmaleimide (MEM). Chromic acid degradation of phycocyanin at 100°C yields MES from ring I, HA from rings II and III, and MEM from ring IV of phycocyanobilin [8,13]. At 25°C, only HA (ring II) and MEM (ring IV) are released from phycocyanin. MES (ring I) and HA (ring III) remain bound to the apoprotein [8,13].

3. Results

The elution pattern of amino acids and radioactivity in hydrolysates of carboxyamidated and non-carboxyamidated ^{35}S-labeled phycobiliproteins is shown in fig.2. The recovery of radioactivity was 92.4% ± 3.0% and of this, 88.7% ± 4.7% cochromatographed with cysteine, CM-cysteine and methionine. Incomplete evacuation of hydrolysis tubes can result in partial conversion of cysteine and methionine into cysteic acid and methionine sulfoxide, respectively. Methionine sulfoxide elutes with CM-cysteine under the conditions of amino acid analysis employed. In non-carboxyamidated samples, significant quantities of radioactivity were not observed in the region of the chromatograms where cysteic acid and methionine sulfoxide elute. Similarly, cysteic acid was not observed on chromatograms of carboxyamidated samples suggesting that the radiolabeled CM-cysteine peaks were not erroneously high due to the presence of radiolabeled methionine sulfoxide.

The chromophore composition, cysteine composition and total radioactivity in the cysteine and CM-cysteine peaks in carboxyamidated phycobiliproteins is shown in table 1. In phycocyanin and allophycocyanin, 65% of the cysteine residues (i.e., 2 of 3) were recovered as CM-cysteine and thus could not participate in a thioether bond. Carboxyamidation of the single cysteine residue in the α subunit of phycocyanin was nearly complete (77% CM-cysteine). Fifty percent of the cysteine residues in the β subunit of phycocyanin were carboxyamidated. This indicates that the cysteine residue in phycocyanin which is available to participate in a thioether bond is present in the β subunit. Since 2 out of 3 cysteine residues in allophycocyanin were carboxyamidated, and this phycobiliprotein contains 2 covalently attached chromophores, only one chromophore could be attached by a thioether linkage involving the free sulfhydryl group of the available cysteine residue.

Five mg phycocyanin was incubated in carboxyamidation buffer with or without subsequent addition of iodoacetamide as described in Materials and methods. Half the reaction mixture was adjusted to pH 2.0 with conc. HCl and extracted with chloroform. Phycocyanobilin was not detected in the chloroform extract of phycocyanin incubated with or without iodoacetamide. The remaining reaction mix-

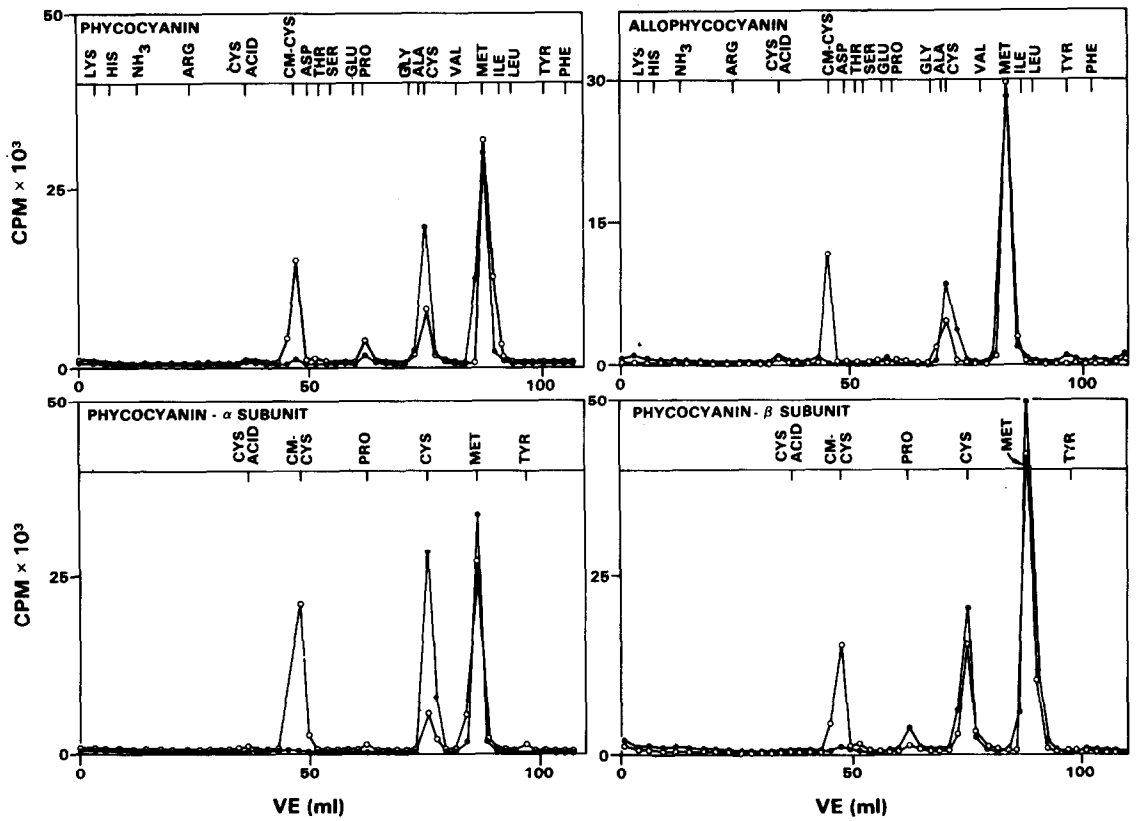

Fig.2. Amino acid analysis of ^{35}S-labeled phycobiliproteins. The data show the elution profile of radioactivity in the hydrolysate of a 0.1 mg sample of carboxyamidated (—○—○—) and non-carboxyamidated (—●—●—) phycobiliprotein. Protein concentrations were determined by quantitative amino acid analysis. Since limited quantities of radiolabeled allophycocyanin were available, unlabelled allophycocyanin was added prior to purification on ion-exchange chromatography columns and amino acid analysis. Consequently, the total radioactivity in cysteine and CM-cysteine in phycocyanin and allophycocyanin cannot be compared directly.

Table 1

Chromophore composition, cysteine composition, and radioactivity associated with cysteine and CM-cysteine in carboxyamidated phycobiliproteins from *Cyanidium caldarium*

Carboxyamidated phycobiliproteins	Chromophore composition[a]	Cysteine composition[a]	Radioactivity[b] (cpm × 10^4)		Radioactivity ratio
	Residues	Residues	Cys	CM-Cys	Cys:CM-Cys
Phycocyanin	3	3	1.08	1.87	1.0 : 1.7
α Subunit	1	1	0.71	2.35	1.0 : 3.3
β Subunit	2	2	2.08	1.92	1.0 : 0.9
Allophycocyanin[c]	2	3	0.69	1.16	1.0 : 1.7

[a]The chromophore composition and cysteine composition have been described previously [9,10]

[b]The radioactivity represents the cpm/0.1 mg protein. Protein concentrations were determined by quantitative amino acid analysis

[c]Since limited quantities of radiolabeled allophycocyanin were available, unlabeled allophycocyanin was added prior to purification on ion-exchange chromatography columns and amino acid analysis

ture was dialyzed against distilled water and chromatographed on a 1.25 cm × 30 cm Sephadex G-100 column equilibrated and developed in 0.05 M potassium phosphate buffer, pH 7.0. The absorption spectrum of non-carboxyamidated phycocyanin in column eluate was nearly the same as that before incubation. The spectral properties of carboxyamidated phycocyanin were irreversibly altered.

Chromic acid degradation of modified and unmodified phycocyanin β subunit yielded only MEM and HA at 25°C and MES, HA and MEM at 100°C. The absence of MES (derived from ring I) in the digest of carboxyamidated β subunit at 25°C clearly demonstrates that the carboxyamidation procedure did not cleave bilin–apoprotein linkages involving ring I of phycocyanobilin.

4. Discussion

Previous work has indicated that phycocyanobilin may be attached to apoprotein by a thioether bond between cysteine and the ethylidine group of ring I (fig.1). This conclusion is based on the identification of cysteine in chromopeptides isolated from phycocyanin after incubation with proteolytic enzymes [5,6]. The results in table 1 show that more cysteine residues were susceptible to carboxyamidation in the *C. caldarium* phycobiliproteins than would be expected if each phycocyanobilin chromophore were covalently attached to the polypeptide subunits by a thioether linkage.

The efficacy of these experiments depends on several factors. Carboxyamidation of 'free' cysteine must be complete. Carboxyamidation of cysteine in ribonuclease A was complete under our conditions. Incomplete carboxyamidation of cysteine in phycobiliproteins would further reduce the number of cysteine residues available to participate in bilin–apoprotein linkages.

The carboxyamidation procedure must leave the linkages intact. The absence of chloroform extractable chromophore after incubation of phycocyanin in carboxyamidation buffer with or without iodoacetamide indicates that bilin–apoprotein linkages were not cleaved during the procedure.

There is indirect evidence suggesting a second (ester) linkage between a carboxyl group on the chromophore and serine in apoprotein [1]. It is possible that the carboxyamidation procedure cleaves one but not both linkages such that phycocyanobilin is not released. This seems unlikely since the absorption spectrum of phycocyanin (which depends on both covalent and non-covalent interactions) could be completely restored after incubation in carboxyamidation buffer (minus iodoacetamide).

The effect of iodoacetamide on the thioether linkage cannot be adequately evaluated by spectral criteria because the spectral properties of carboxyamidated phycocyanin are different from unmodified phycocyanin. However, the integrity of bilin–apoprotein linkages involving ring I following carboxyamidation was unequivocally established by the absence of MES in the chromic acid digest of carboxyamidated β subunit at 25°C.

In summary, the present results demonstrate that ring I of both phycocyanobilin residues in the phycocyanin β subunit must be linked to apoprotein, one through cysteine and the other to a different amino acid. Similarly, cysteine is involved in some, but not all of the bilin–apoprotein linkages in *Cyanidium caldarium* phycobiliproteins.

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