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BILIN–APOPROTEIN BONDS IN CRYPTOMONAD PHYCOERYTHRIN

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Key Word Index—*Rhodomonas*; cryptomonad; phycoerythrin; amino acid analysis; bilin–apoprotein bond; biliproteins.

Abstract—Analysis of a homogeneous chromopeptide prepared by proteolytic digestion of *Rhodomonas* phycoerythrin revealed four amino acids: alanine, cysteine, serine, glutamic acid. The chromopeptide was also analyzed for *N*- and *C*-terminii and free thiol (cysteine) and hydroxyl (serine) groups. These results indicated that the bilin in cryptomonad phycoerythrin is bound through both propionic acid side-chains to serine (ester bond) and glutamic acid (amide bond) in the apoprotein.

INTRODUCTION

THE PHOTOSYNTHETICALLY active algal biliproteins (phycocyanins and phycoerythrins) have linear tetrapyrrolic prosthetic groups (bilins) which are bound to the apoprotein by covalent bonds.[1,2] Analysis of *C*-phycocyanin (for nomenclature designation see [3]) chromopeptides[4] has suggested two bonds between bilin and apoprotein: one, an ester bond between the hydroxyl group of the lactim form of ring I of the bilin and a carboxyl group of aspartic acid in the apoprotein and two, a thioether-type of bond between the ethylidene side-chain on ring I of the bilin and the thiol group of cysteine.

From analysis of *R*-phycoerythrin chromopeptides Fujiwara[5,6] concluded that the bilin might be linked to the apoprotein through a thioether bond involving cysteine. In phycoerythrin and phycocyanin from both blue–green and red algae it was shown[2] that covalent bonds between bilin–apoprotein involved ring I and either ring II or III of the bilin. After analysis of *B*-, *C*- and *R*-phycoerythrin chromopeptides, Killilea and O'Carra[7] proposed that the bilin was linked to serine and glutamic acid in the apoprotein: serine could be bound to the proprionic acid side-chain on ring II or III of the bilin to form an ester bond and glutamic acid could be bound to the ring I pyrrolone nitrogen through the γ-carboxyl group to form an amide-type bond. This model substantiates the results of Rüdiger and O'Carra.[2]

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[1] H. W. SIEGELMAN, D. J. CHAPMAN and W. J. COLE, in *Porphyrins and Related Compounds* (edited by T. W. GOODWIN), p. 107, Academic Press, New York (1968).
[2] W. RÜDIGER and P. O'CARRA, *Europ. J. Biochem.* **7**, 509 (1969).
[3] C. O'hEOCHA, *Ann. Rev. Plant Physiol.* **16**, 415 (1965).
[4] H. L. CRESPI and U. H. SMITH, *Phytochem.* **9**, 205 (1970).
[5] T. FUJIWARA, *J. Biochem. Tokyo* **44**, 723 (1957).
[6] T. FUJIWARA, *J. Biochem. Tokyo* **48**, 317 (1960).
[7] S. D. KILLILEA and P. O'CARRA, *Biochem. J.* **110**, 14P (1968).

Because the biliproteins of cryptomonad algae differ from those of blue-green and red algae[3] we decided to prepare chromopeptides from a cryptomonad phycoerythrin to determine if the bilin–apoprotein linkages also differed. It was found necessary to develop new purification methods for these chromopeptides. Criteria for what constituted a desirable chromopeptide are outlined in the Discussion. Appropriate analyses of homogeneous chromopeptides indicated which two rings of the bilin were linked to the apoprotein, as well as the apoprotein amino acids involved in these two linkages.

RESULTS

Phycoerythrin after the ammonium sulfate gradient-elution chromatography had an extinction ratio ($E_{\lambda max}/E_{275}$) of less than 3. Further purification by gel filtration chromatography increased this ratio to 3·5–5·0. As *Rhodomonas* contains only phycoerythrin,[8] contamination from other biliproteins was not a problem and absorption spectra of this purified phycoerythrin showed that no other conjugated tetrapyrrole-containing proteins were present. Phycoerythrin with an extinction ratio of $>4{\cdot}5$ is considered pure in terms of one of the criterion conventionally used in biliprotein biochemistry (e.g. see [9]) and chromopeptides were prepared exclusively from this material. *Rhodomonas* phycoerythrin contains only one type of bilin[8] so it was assumed that all chromopeptides contained a bilin of identical structure.

Chromopeptide Purification

Using 1-pentanol-soluble material from proteolytic digests of *Rhodomonas* phycoerythrin a number of colored zones were separated by gel filtration on Bio-Gel P2. A brown zone was always eluted first and discarded; normally five red zones followed. Each fraction of the last-eluted red zone was scanned in the UV region of the spectrum and those fractions with a single maximum at 304–308 nm (leading edge) were discarded while those fractions with maxima at 268–270 and 330 nm (trailing edge) were combined and are henceforth referred to as Fraction 8. Sometimes less than five red zones were eluted from the Bio-Gel column; nonetheless, the last eluted red zone could still be divided into two portions based upon the UV spectra as just described.

Sepration of Fraction 8 by analytical TLC on acidic alumina in ethyl formate–formic acid revealed three red bands (R_fs 0·90, 0·78, 0·47) as well as a minor brown band which ran just behind the solvent front. A colorless contaminating nitrogenous compound was visualized (R_f 0·95) after monitoring chromatograms with the tertiary-butyl hypochlorite procedure.[10] This separation of Fraction 8 was verified because no additional colored or colorless bands were found using the following thin-layer systems: (a) 1-butanol–pyridine–water (3:1:1) on basic alumina; (b) 1-pentanol–water–methanol (4:5:3) plus 1% ammonium hydroxide (by vol) on neutral alumina; (c) ethyl formate–formic acid (3:1) on neutral alumina; and (d) 1-butanol–glacial acetic acid–water (3:1:1) on neutral alumina.

The principal chromopeptide (R_f 0·47 on acidic alumina in ethyl formate–formic acid) in Fraction 8 had absorption maxima at 545, 330 and 268 nm (Fig. 1). This chromopeptide was regarded as a homogeneous substance because it could not be further fractionated by using any of the thin-layer systems described above and because the amino acid residues in

[8] D. J. CHAPMAN, W. J. COLE and H. W. SIEGELMAN, *Phytochem.* **7**, 1831 (1968).
[9] J. J. LEE and D. S. BERNS, *Biochem. J.* **110**, 457 (1968).
[10] R. H. MAZUR, B. W. ELLIS and P. S. CUMMARATA, *J. Biol. Chem.* **237**, 1619 (1962).

this chromopeptide occurred in equimolar amounts (molar ratios alanine 1·00: cysteine 1·01: glutamic acid 1·12: serine 1·01). It appears that the bilin in this chromopeptide is identical to the bilin in purified starting phycoerythrin because the visible absorption spectra of both are very similar (Fig. 1).

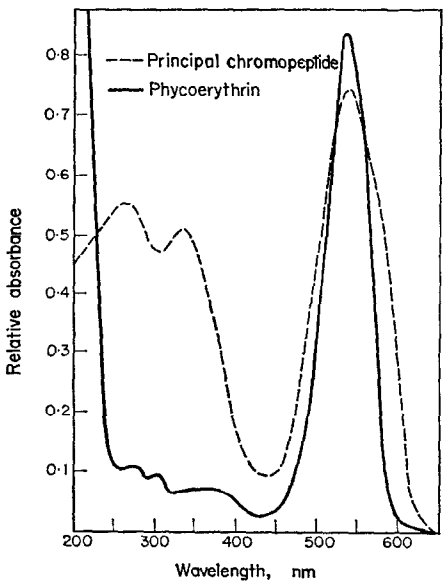


FIG. 1. SPECTRA OF PURIFIED *Rhodomonas* PHYCOERYTHRIN IN 100 mM PHOSPHATE BUFFER, pH 6·8 AND THE PRINCIPAL CHROMOPEPTIDE FROM FRACTION 8 IN 200 mM HOAc.

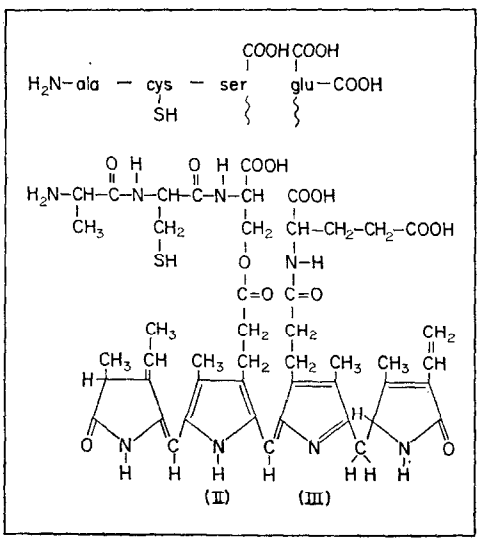


FIG. 2. ONE OF THE TWO PROPOSED STRUCTURES FOR APOPROTEIN–PHYCOERYTHROBILIN LINKAGES IN CRYPTOMONAD TYPE 1 PHYCOERYTHRIN.

Quantitative and qualitative amino acid analysis of this homogeneous chromopeptide revealed four amino acid residues: alanine, serine, glutamic acid and cysteine, all present in equimolar amounts. Because the hydrolysis tubes had been vigorously degassed, cysteine quantitated well. By use of the spray reagent *p*-dimethylaminobenzaldehyde[11] it was established that trytophan was absent in the purified chromopeptide; a test spot of tryptophan which had been applied to a thin-layer plate containing purified chromopeptide gave the characteristic color with this reagent. The test reagent does react with bilenes but does not react with biladienes such as phycoerythrobilin.

An *N*-terminal determination by the dansyl-chloride method revealed only *N*-terminal alanine. Co-chromatography with the dansyl-derivatives of alanine, glutamic acid, serine and cysteine in four solvents[12] confirmed that alanine was the only amino acid residue yielding a dansyl-derivative and hence that it was the only *N*-terminus.

Acid-catalyzed hydrazinolysis and dinitrophenylation[13] established that both serine and glutamic acid were *C*-terminii. Co-chromatography of the experimentally acquired DNP-derivatives with authentic DNP-serine and DNP-glutamic acid established their nature and confirmed their identity as the only two *C*-terminii. Because cysteine is probably destroyed

[11] K. K. REDDI and E. KODICEK, *Biochem. J.* **53**, 286 (1953).
[12] K. R. WOODS and K. WANG, *Biochim. Biophys. Acta* **133**, 369 (1967).
[13] J. L. BAILEY, *Techniques in Protein Chemistry*, 2nd Edition, Elsevier, Amsterdam (1967).

by acid-catalyzed hydrazinolysis it is possible that it was *C*-terminus, but this possibility does not interfere with interpretation of results as regards the nature of bilin–apoprotein linkages in crytomonad phycoerythrin (see Discussion).

The chromic anhydride-sulphuric acid test[14] for a free primary or secondary hydroxyl group was negative indicating that the hydroxyl group of serine was bound. A control determination on a solution of serine (2×10^{-6} mol) showed that the test was useful at this concentration. Using a pure chromopeptide (2×10^{-6} mol) prepared from phycoerythrin which had only been denatured in guanidine–HCl, the sodium nitroprusside test[15] for free sulphydryl groups was positive indicating that the thiol group of cysteine was not bound. The yield of thiol by this assay approached the calculated value of one sulphydryl group per chromopeptide and a control determination on a solution of cysteine (containing 2×10^{-6} mol) showed that this concentration exceeded the limit of detection.

DISCUSSION

The preparation of chromopeptides from a cryptomonad phycoerythrin was made possible by our ability to obtain kilogram quantities of *Rhodomonas* and thereby having abundant phycoerythrin for our experiments. Fractionation of phycoerythrin from small quantities of *Rhodomonas* has previously been reported;[16,17] however, no criterion was presented by which to evaluate the purity of these preparations. The methods of phycoerythrin purification presented in this paper yielded a preparation with an extinction ratio $>3{\cdot}5$. Although phycoerythrin is the dominant protein component it is now known[18] that phycoerythrin preparations with extinction ratios of even greater than 4·5 contain a small amount of colorless material. However, to obtain homogeneous chromopeptides it was not essential that the starting phycoerythrin preparations be absolutely pure but it was essential that there be no other conjugated tetrapyrrole-containing proteins in the phycoerythrin that will be digested to obtain chromopeptides. Phycoerythrin with an extinction ratio of greater than 4·5 does not contain other biliproteins, chlorophylls or cytochromes (see Results). The small quantity of colorless material in the starting phycoerythrin preparations has no significance in this research because large amounts of colorless and colored peptides are normally generated by enzymatic digestion of even a pure colored protein. However, because it was known that colorless peptides would be generated it was considered mandatory to scan all TLCs for contaminating colorless nitrogenous compounds.

Determination of the types of bilin–apoprotein linkages and identification of those amino acids joined directly to the bilin by analysis of enzymatically prepared chromopeptides requires a small, homogeneous chromopeptide (4–8 residues) of known amino acid composition. Identification of the bilin–apoprotein bonds can then be achieved by appropriate analyses of the amino acid residues present in a particular chromopeptide, hence it was necessary to perform tests for thiol and hydroxyl side-groups and *N*- and *C*-terminal determinations.

Following proteolytic digestion of phycoerythrin the 1-pentanol-soluble material was

[14] R. L. SHRINER, R. C. FUSON and D. Y. CURTIN, *The Systematic Identification of Organic Compounds*, 5th Edition, p. 125, Wiley, New York (1965).
[15] K. G. KREBS, D. HEUSSER and H. WIMMER, *Thin-layer Chromatography* (edited by E. STAHL), 2nd Edition, p. 890, Springer, Berlin (1969).
[16] C. O'hEOCHA and M. RAFTERY, *Nature, Lond.* **184,** 1049 (1959).
[17] F. T. HAXO and D. C. FORK, *Nature, Lond.* **184,** 1051 (1959).
[18] C. BROOKS, unpublished observations.

fractionated by Bio-Gel P2 chromatography because chromopeptides containing 1–8 amino acid residues would have MWs well within the filtration limits of this gel. The effective operating range of Bio-Gel P2 is about 200–1800 and assuming an average amino acid residue MW of 121 g/mol and a bilin MW of about 600,[1] these chromopeptides would have MWs from about 750–1550. Fraction 8 was judged by its elution volume to have the desired amino acid composition and therefore was chosen for purification and further analysis. Separation of Fraction 8 was finally achieved by TLC on acidic alumina in ethyl formate–formic acid. Subsequent chromatography of the principal chromopeptide in Fraction 8 in other systems (see Results) revealed no other nitrogenous components. The claim of purity for this chromopeptide is further substantiated by the amino acid analysis showing only four residues present in equimolar amounts. To determine the apoprotein amino acids joined directly to the bilin it was essential to know which amino acid side-groups (appropriate to this chromopeptide amino acid composition) were free and which amino acids were *N*- and *C*-terminii.

The sodium nitroprusside test[15] demonstrated that the sulphydryl group of cysteine was free eliminating the possibility of a thioether bond through either the vinyl or ethylidene side-chain on the bilin. This result is in keeping with unpublished observations that certain proteolytic enzymes (e.g. subtilisin) cleave the bilin from the apoprotein. This would not be expected if a thioether linkage were present. The chromic anhydride–sulphuric acid test[14] showed that the hydroxyl of serine was bound. This indicates a seryl ester linkage through one of the propionic acid side-chains on the bilin. This conclusion agrees with both enzymatic[19] and chemical cleavage[2,3] data which also indicated an ester bond between bilin and apoprotein involving the propionic acid side-chain on ring II or III of the bilin. The results of *N*- and *C*-terminal determinations indicated that glutamic acid is joined to the other propionic acid side-chain. Glutamic acid and serine were *C*-terminii but as alanine was the only *N*-terminus this shows that the amino group of glutamic acid is covalently bound to the bilin and also that glutamic acid is a bound isolated residue. It is possible that the amino group of glutamic acid might be linked to ring I of the bilin as suggested by Crespi and Smith[4] for aspartic acid in *C*-phycocyanin, however, Rüdiger and O'Carra[3] maintain that if the hydroxyl group at ring I were linked to apoprotein, this ring would be forced into the lactim form but according to them, Chapman *et al.*[20] and Crespi *et al.*[21] the bilin exists in the lactam (carbonyl group) form. These arguments add further support to the proposal of an amide bond involving the amino group of glutamic acid and a propionic acid side-chain on the bilin. Serine could not be the bound isolated residue because linkage via the hydroxyl group would have resulted in a second *N*-terminus and linkage via the amino group would have left the hydroxyl group free. Therefore, the results of appropriate analyses of a homogeneous chromopeptide from *Rhodomonas* phycoerythrin establish that the bilin is joined through both propionic acid side-chains to serine and glutamic acid in the apoprotein to form an ester and an amide bond, respectively.

The sequence of the alanine, cysteine and serine residues near the bilin is not known with certainty. Locating cysteine is the problem; it could have been a *C*-terminal residue since it probably would have been lost in the acid-catalyzed hydrazinolysis and dinitrophenylation procedure.[13] If it had been a *C*-terminus it is difficult to establish its position in view of the other results, particularly as it is not linked to the bilin. We have assumed that cysteine

[19] H. W. SIEGELMAN, D. J. CHAPMAN and W. J. COLE, *Arch. Biochem. Biophys.* **122,** 261 (1967).
[20] D. J. CHAPMAN, W. J. COLE and H. W. SIEGELMAN, *J. Am. Chem. Soc.* **89,** 5976 (1967).
[21] H. L. CRESPI, U. SMITH and J. J. KATZ, *Biochemistry* **7,** 2232 (1968).

was not *C*-terminal and have assigned it to the alanine–serine peptide to give alanine–cysteine–serine–bilin–glutamic acid (Fig. 2); it is possible that the alanine–serine peptide is attached to ring III and that glutamic acid is attached to ring II of the bilin. It must be emphasized that sequencing the tetrapeptide was not the principal objective of this research but rather it was the development of methods for separating and purifying chromopeptides from a phycoerythrin and determination of those apoprotein amino acids linked directly to the bilin as well as the chemical nature of these bonds.

After analysis of *B*-, *C*- and *R*-phycoerythrin chromopeptides Killilea and O'Carra[7] proposed two linkages between bilin and apoprotein but unfortunately they failed to publish quantitative amino acid data so one can not determine the purity of the chromopeptides analyzed. It was not reported whether the side-groups of aspartic acid, glutamic acid, serine and threonine were bound or free. Killilea and O'Carra[7] concluded that the bilin was linked to the apoprotein through glutamic acid and serine, since serine could form an ester bond with a propionic acid side-chain on the bilin and the γ-carboxyl group of glutamic acid could be linked to the pyrrolone ring I nitrogen of the bilin. In the present investigation, if the γ-carboxyl group of glutamic acid were linked to ring I of the bilin to form an amide bond, two *N*-terminii would have been recovered. Because this was not the case, the present placement of glutamic acid being linked to the propionic acid side-chain of the bilin is confirmed.

The model of bilin-apoprotein linkages proposed by Killilea and O'Carra[7] is apparently representative of phycoerythrins from blue–green and red algae as it agrees with the one proposed by Rüdiger and O'Carra.[2] However, their proposal may not apply to phycoerythrins from cryptomonad algae especially because crytpomonad phycoerythrins differ from those in the other two algal groups (for review, see [3]). The uniqueness of cryptomonad phycoerythrins is further emphasized by our results which show that the bilin–apoprotein linkages also differ from those of any other phycoerythrin investigated.

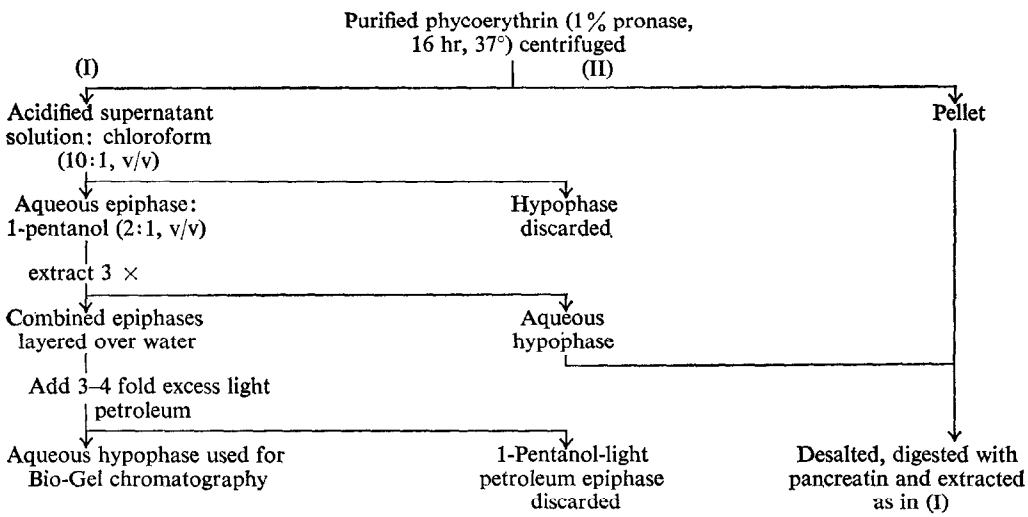
EXPERIMENTAL

Phycoerythrin purification. Cryptomonad phycoerythrin was extracted from *Rhodomonas* strain 3-C cultured autotrophically in D-base medium[22] using a 200-l. mass culture apparatus.[23] After 8–10 days the cells were harvested with a Szent–Gyorgyi–Blum continuous-flow device. Using a Waring blender the pellets were homogenized in an approx. equal vol. of 100 mM potassium phosphate buffer, pH 6·8, and the slurry was then frozen and thawed twice in succession. Cellular debris was removed by centrifugation (10000 *g*, 120 min) and the pellet was washed with 100 mM phosphate buffer until it was green. All supernatant solutions were combined and the phycoerythrin precipitated after making the solution 90% (w/v) saturated with respect to $(NH_4)_2SO_4$. The precipitated phycoerythrin was collected by centrifugation and then fractionated by $(NH_4)_2SO_4$ gradient-elution column chromatography.[1] All fractions having an extinction ratio ($E_{\lambda max}/E_{280}$) of one or more were combined and the phycoerythrin concentrated by precipitation with $(NH_4)_2SO_4$. The precipitate could be stored at 4° in 90% sat $(NH_4)_2SO_4$ for up to 6 months. The phycoerythrin was further purified by gel filtration column chromatography. The precipitated phycoerythrin was dissolved in 40 ml of 100 mM phosphate buffer, pH 6·8, and applied to a column (3·5 × 55 cm) of Sephadex G75; the column was eluted (40 ml/hr) with phosphate buffer and the phycoerythrin-containing fractions were collected. The phycoerythrin was eluted as a single zone and those fractions with extinction ratios of >4·5 were combined and the phycoerythrin precipitated with $(NH_4)_2SO_4$. The precipitated phycoerythrin was stored in a sat. $(NH_4)_2SO_4$ solution at 4° for up to 2 weeks.

Chromopeptide preparation. The precipitated phycoerythrin was dissolved in distilled water and dialyzed at 4° against a 200 × excess of distilled water. The solution was then made 8 M with respect to guanidine-HCl and stirred under N_2 for several hours. The disulfide bonds of the denatured phycoerythrin were reduced with an excess of 2-mercaptoethanol and then aminoethylated with ethyleneimine. The aminoethylated phycoerythrin was dialyzed against a 200 × excess of distilled H_2O, concentrated under vacuum and then

[22] L. PROVASOLI, J. J. MCLAUGHLIN and M. R. DROOP, *Arch. Mikrobiol.* **25**, 392 (1965).
[23] H. LYMAN and H. W. SIEGELMAN, *J. Protozool.* **14**, 297 (1967).

phosphate buffer, pH 7·2, was added until the buffer concentration was 50 mM. The steps of reduction and aminoethylation were elimianted in the preparation of chromopeptide used for total amino-acid analysis and free thiol determinations. After degassing, the phycoerythrin solution was bubbled with N_2 and incubated at 37° with pronase (enzyme: substrate ratio 1:100, w/w). The chromopeptide extraction procedure is presented in Scheme 1. After 16 hr the digest was clarified by centrifugation and the pellet was saved for digestion with pancreatin. The supernatant was diluted with an equal vol. of distilled water, made to pH 2–3 with dil. HCl and then extracted once with 0·2 vol. $CHCl_3$; the hypophase was discarded. The remaining aqueous epiphase was extracted with 0·5 vol. of 1-pentanol; this procedure was repeated 2 ×. The 1-pentanol extracts were combined, layered over a small vol. of distilled water, and the 1-pentanol-soluble material transferred to the distilled water by the addition of a 3–4 fold excess of light petroleum. The aqueous hypophase remaining after the third 1-pentanol extraction was used to extract the pellet obtained after the pronase digestion. This colored extract was desalted by gel filtration chromatography on Sephadex G25 using distilled water as elutant. The eluted colored zone was concentrated under vacuum and adjusted to 50 mM with 1 M Tris buffer, pH 7·8. The degassed solution was bubbled with N_2 and incubated for 16 hr at 37° with pancreatin (enzyme: substrate ratio 1:50, w/w), after which the enzymatic digest was centrifuged; the pellet being discarded. The supernatant was fractionated by the above procedure to prepare 1-pentanol-soluble material which was also transferred to water and then combined with the homologous fraction obtained after the pronase digestion. The red aqueous solution was concentrated by rotary evaporation at 25–30° by the addition of EtOH.



SCHEME 1. EXTRACTION OF CHROMOPEPTIDES FROM PURIFIED PHYCOERYTHRIN.

Chromopeptide purification. The red aqueous solution (10 ml) was fractionated by gel filtration chromatography on Bio-Gel P2. The column (3·2 × 36 cm) had a flow rate of 30 ml/hr and 5 ml fractions were collected. The trailing portion of the last eluted red zone (Fraction 8) was purified by analytical TLC on acidic alumina (activated by heating for 30 min at 110°). Fraction 8 was taken to near-dryness, transferred to EtOH and applied to the chromatograms. The acidic alumina plates were developed at room temp. in the dark in HCO_2Et–HCO_2H (17:6), and the principal colored band (R_f 0·47) was eluted with 200 mM HOAc. A portion of every chromatogram was developed by the tertiary-butyl hypochlorite procedure[10] to visualize colorless contaminating nitrogenous compounds.

Amino acid analysis. The principal homogeneous chromopeptide from Fraction 8 was used for an amino acid analysis. The dimethyl ester of phycoerythobilin has an extinction coefficient ($E^{1\%}_{1cm}$) of 360,[1] therefore, the chromopeptide was assumed to have an extinction coefficient of 350. The chromopeptide (0·3 mg) in HOAc

was taken to dryness under vacuum, dissolved in a minimal vol. of 6 M HCl, thoroughly degassed and then hydrolyzed at 110° for 24 hr. The hydrolysis mixture was then dried under vacuum and the residue taken up in 200 mM citrate buffer, pH 2·2 containing 200 mM sodium ion. Qualitative and quantitative amino acid determinations were performed with a Bio-Cal 200 automatic amino acid analyzer.

Tryptophan determination. After final purification of the chromopeptide by analytical TLC, a tryptophan determination was performed by spraying a portion of a thoroughly dried plate with 0·5 g of *p*-dimethylaminobenzaldehyde in 100 ml 95% EtOH containing 2 ml of concentrated HCl.[11] Sufficient chromopeptide was present to assure that tryptophan, if present, was above the level of sensitivity (1 μg) of the reagent employed.

N- *and C-terminal determinations.* An aliquot (2×10^{-7}mol) of homogeneous chromopeptide was subjected to an *N*-terminal determination by dansylation. The purified chromopeptide was adjusted to pH 7·5 by the addition of $NaHCO_3$ and 9·6 mg of dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) in acetone (6 mg/ml) was added and the mixture was placed in the dark for several hours. It was then dried under vacuum and the residue dissolved in 6 M HCl, degassed and hydrolyzed at 110° for 24 hr. The hydrolyzed mixture was dried and the residue dissolved in Me_2CO–HOAc (3:2) and then two-dimensional TLC on polyamide was performed.[12] Identification of the *N*-terminal residue was varified by one-dimensional co-chromatography with known dansyl-derivations on polyamide in four solvents.[12] An aliquot (2×10^{-7} mol) of homogeneous chromopeptide was subjected to a *C*-terminal determination by acid-catalyzed hydrazinolysis[13] and dinitrophenylation. Two and one-dimensional co-chromatography using the appropriate DNP (dinitrophenyl) derivatives was used to identify the *C*-terminal residues.

Primary or secondary hydroxyl group and thiol determinations. An aliquot (2×10^{-6} mol) of pure chromopeptide was tested for the presence of the primary alcohol side-group of serine with a CrO_3–H_2SO_4 reagent.[14] An aliquot (2×10^{-6} mol) of pure chromopeptide was tested for the presence of SH of cysteine with sodium nitroprusside.[15] A pure chromopeptide prepared from phycoerythrin which had only been denatured in guanidine–HCl was brought to pH 8·4 with dilute NH_4OH. This solution was placed in both compartments of a spectrophotometer; 0·2 ml of a freshly prepared sodium nitroprusside reagent[15] was added to the sample compartment cell and the absorbance increase at 519 nm was recorded. The color change could also be perceived visually.

Note added in proof. It is possible, but very unlikely, that cysteine forms an amide bond to ring III of the bilin.

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