Phytochemistry, 1970, Vol. 9, pp. 205 to 212. Pergamon Press. Printed in England.

THE CHROMOPHORE–PROTEIN BONDS IN PHYCOCYANIN*

HENRY L. CRESPI and URSULA H. SMITH

Chemistry Division, Argonne National Laboratory, Argonne, Illinois 60439, U.S.A.

(*Received* 23 *June* 1969)

Abstract—Peptides formed by the proteolysis of C-phycocyanin were purified by TLC and analyzed for amino acid content. The data suggest that phycocyanobilin is linked to apophycocyanin through two bonds, one an ester bond involving the carboxyl group of an aspartic acid side-chain and the hydroxyl group of the enol form of ring A of the bilin, and the other a thio-ether type of bond from a cysteine side-chain to the bilin side-chain at position 2, a linkage analogous to that in cytochrome c. Liberation of phycocyanobilin from apophycocyanin by alcohols would involve transesterification at the ester bond followed by the formation of the keto form of ring A and elimination of the cysteine side-chain.

INTRODUCTION

PHYCOCYANIN is one of a group of plant chromoproteins important in photosynthesis and plant physiology. Among the members of this group are allophycocyanin, phycoerythrin, and phytochrome. Phycocyanin, allophycocyanin, and phycoerythrin are accessory photosynthetic pigments found in high concentration and in many algae.[1] Phytochrome is a photoregulator of plant growth and development[2] and is presumed to have a chromophore similar to that of other members of the group.[3] The structure of the prosthetic group of phycocyanin, phycocyanobilin (PCB), is that of a linear tetrapyrrole. Details of its structure have recently been determined in this and in other laboratories.[4–10] The structure of the prosthetic group of phytochrome remains to be determined.[3]

The question now arises as to the mode of covalent attachment of phycocyanobilin and phycoerythrobilin to apoprotein. The bonding of these bilins to apoprotein must be compatible with the experimental observations that bilin is liberated from apoprotein, and extractable into chloroform, by refluxing in neutral solutions of alcohols, by treatment with aqueous acid or base, and by treatment with the proteolytic enzyme Nagarse.[3, 5, 11–13] The

* Based on work performed under the auspices of the U.S. Atomic Energy Commission.

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[4] H. L. CRESPI, L. J. BOUCHER, G. NORMAN, J. J. KATZ and R. C. DOUGHERTY, *J. Am. Chem. Soc.* **89**, 3642 (1967).
[5] H. L. CRESPI, U. SMITH and J. J. KATZ, *Biochemistry* **7**, 2232 (1968).
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deduction from these data is that an ester bond is being broken. Killilea and O'Carra[14] suggest that phycoerythrobilin is doubly linked to apoprotein: first by an ester bond from serine to a propionic acid side-chain of the bilin, and second, by a bond from the γ-carboxyl group of glutamic acid to the lactam grouping of ring A of the bilin. The pattern of amino acids found by Killilea and O'Carra[14] in the chromopeptides from phycoerythrin is somewhat similar to that found by Crespi *et al.*[5] in work with peptides from phycocyanin. However, Crespi *et al.*[5] questioned the possibility of an ester linkage involving a propionic acid side-chain of the bilin, since the PCB liberated by methanolysis of phycocyanin is essentially the free diacid. Similarly, we have observed that phycoerythrobilin is liberated by methanol as a diacid.[14] We wish to report here work on the composition of peptides obtained by hydrolysis of phycocyanin that suggests two covalent bonds between PCB and apoprotein. One bond may be an ester linkage between an aspartic acid side-chain of the protein and the hydroxyl group at ring A of the enol form of the bilin; a second bond is a thio-ether link between a cysteine side-chain of the protein and the methine carbon of the ethylidene group at position two in ring A of PCB (see Fig. 1).

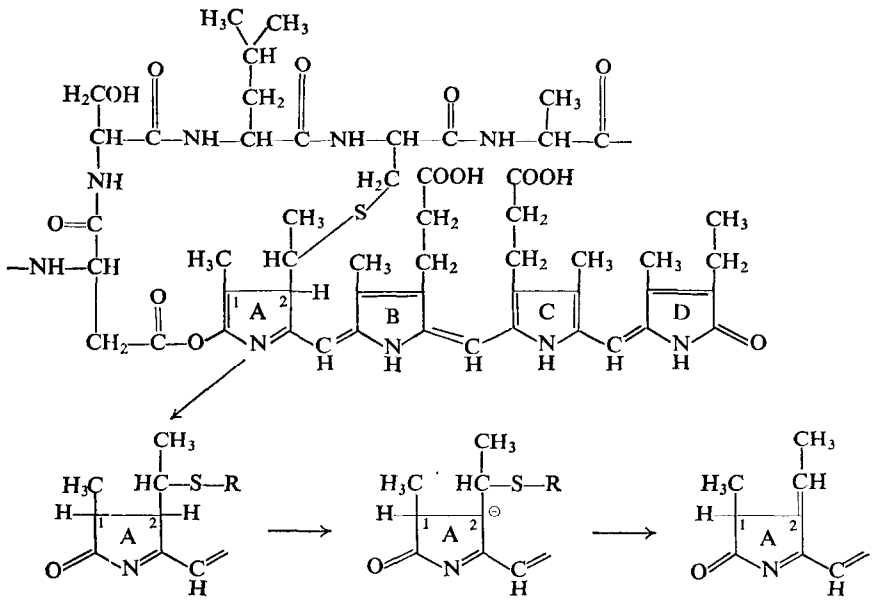

FIG. 1. THE PROPOSED MODE OF ATTACHMENT OF PHYCOCYANOBILIN IN *C*-PHYCOCYANIN. The upper, full structural formula shows the two bonds from apoprotein to ring A of the bilin. Methanolysis of the ester linkage would cause ring A to return to its keto form, leading to activation at position 2 and complete liberation of PCB.

RESULTS AND DISCUSSION

The chloroform extract (representing about 5 per cent of the total PCB in the digest) of phycocyanin digested with Nagarse showed two blue bands on chromatography in System A,* plus small amounts of material of other colors. The faster moving of the blue bands (R_f 0·6) was free PCB. The slower moving material (R_f about 0·3) proved to be a chromopeptide of variable composition, either PCB–leucine–cysteine or PCB–alanine–leucine–cysteine (Table 1). The butanol-soluble peptides comprised the bulk of the PCB in the digest.

* For solvent key, see Experimental.

[14] H. L. CRESPI and J. J. KATZ, *Phytochem.* **8**, 759 (1969).

TABLE 1. AMINO ACID COMPOSITION OF THE CHLOROFORM-SOLUBLE CHROMOPEPTIDE FROM PHYCOCYANIN

	Micromoles × 10^3 of PCB or amino acid			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Chromatographic solvent:*	A	A	AB	A^2
PCB:†	100 (270)	100 (150)	100 (56)	100 (43)
Amino acids				
Aspartic acid	6	5	—	—
Threonine	3	4	—	—
Serine	12	5	—	—
Glycine	—	6	—	—
Alanine	—	72	156	—
Cysteine‡	123	186	117	42
Valine	10	12	—	—
Methionine	19	—	—	—
Isoleucine	4	5	—	—
Leucine	121	160	119	30

* The letters indicate the systems with which the chromopeptides were purified and the order in which they were used. A superscript 2 means that system was used twice. For key, see Experimental.

† All amino acid values were normalized to a value of 100×10^{-3} micromoles of PCB. The amount of PCB in each sample was determined spectrophotometrically using the absorptivity for free PCB,[5] which will give approximately correct values for the amount of chromopeptide. The experimentally observed amounts of PCB in each sample are given in parenthesis.

‡ The "cysteine" value is in each case the sum of cysteic acid, cystine, cysteine and *S*-carboxymethyl cysteine, which was sometimes present but in very small amounts.

The final neutral, aqueous solution of peptides contained about 40 per cent of the initial amount of PCB in the digest. These peptides were chromatographed first in System A; three blue bands were observed, fraction 1 (R_f 0·11), fraction 2 (R_f 0·20) and fraction 3 (R_f 0·30). (Unless the original digest is extracted with chloroform, interpretation of this chromatogram is made very difficult by the presence of a number of extraneous pigment bands.) When re-chromatographed with System C, fraction 1 gave a single blue band, but fractions 2 and 3 gave two blue bands each, designated 2-1, 2-2 and 3-1, 3-2. Amino acid data obtained with the butanol-soluble peptides is summarized in Tables 2–4. As indicated in these tables, a number of different series of chromatographic purification steps also were used in addition to the series System A→System C described above.

Table 2 presents the results of the analysis of fraction 1 in a variety of solvents. This chromopeptide seemed especially difficult to purify, but the results indicate clearly that this peptide is composed of (Asp Ser Gly Cys Leu) and perhaps alanine or that it is a mixture of this peptide and (Asp Ser Gly Cys Leu Ala). Only traces of the basic amino acids were observed in this fraction. Data obtained with fraction 2 is given in Table 3. Purification of fraction 2 in Systems A and B indicated one blue component associated with (Ala Cys Leu). However, when fractionation was carried to System C, we obtained peptides 2–1 (Ala Cys Leu), and 2-2, cysteine alone.

The analysis of fraction 3 is given in Table 4. This peptide contained cysteine and leucine. (Peptide 3-1 was present in only very small amounts, insufficient for an accurate analysis.) Peptide 3-2 again clearly indicates a (Cys Leu) dipeptide as in the chloroform-soluble material. However, the yield of this peptide is considerably higher than the chloroform-soluble

TABLE 2. AMINO ACID COMPOSITION OF CHROMOPEPTIDES OF FRACTION ONE*

	Micromoles × 10^3 of PCB or amino acid				
	Expt. 40A	Expt. 40B	Expt. 41	Expt. 42	Expt. 46
Chromatographic solvents:	AC	CA	AB	A^2	A^2C
PCB:	100 (72)	100 (77)	100 (196)	100 (83)	100 (141)
Amino acids					
Aspartic acid	57	51	137	28	75
Threonine	—	12	9	—	4
Serine	36	79	135	28	36
Glutamic acid	—	31	15	11	8
Proline	—	—	—	—	—
Glycine	33	47	78	25	28
Alanine	25	22	25	16	26
Cysteine	45	34	65	49	71
Valine	26	4	30	10	33
Methionine	18	6	8	—	33
Isoleucine	22	18	19	11	31
Leucine	43	56	128	42	73
Tyrosine	—	—	4	—	—
Phenylalanine	—	—	8	—	—

* See footnotes to Table 1.

TABLE 3. AMINO ACID COMPOSITION OF CHROMOPEPTIDES OF FRACTION TWO*

	Micromoles × 10^3 of PCB or amino acid			
			Expt. 46	
	Expt. 33	Expt. 42	2–1	2–2
Chromatographic solvents:	AB	A^2	A^2C	A^2C
PCB:	100 (174)	100 (82)	100 (38)	100 (67)
Amino acids				
Aspartic acid	17	11	42	27
Threonine	6	4	21	8
Serine	9	12	52	20
Glutamic acid	17	11	34	23
Proline	—	—	—	—
Glycine	13	10	39	29
Alanine	59	20	252	26
Cysteine	56	42	169	143
Valine	19	—	25	15
Methionine	—	—	5	—
Isoleucine	17	9	15	12
Leucine	46	22	153	21
Tyrosine	—	—	—	—
Phenylalanine	—	—	—	—

* See footnotes to Table 1.

material. It is not clear to us why a small portion of the chromopeptides are soluble in chloroform. Also listed in Table 4 is the result of analysis of a peptide that ran as the middle of three blue components in System C and then as the main component in System A. This chromopeptide is composed of (Asp Ser Cys Leu).

The data of Tables 1–4 indicate that, in phycocyanin, PCB is attached to apoprotein through a peptide of the composition and sequence Gly(Asp Ser)Leu Cys Ala. This sequence is called for by the fact that we have the following chromopeptides: Cys, (Leu Cys), (Leu Cys Ala), (Asp Ser Leu Cys), and (Gly Asp Ser Leu Cys). The data indicates a covalent bond between PCB and the sulfur atom of cysteine. The presence of a cysteine–PCB "peptide" (Table 3) makes unlikely a linkage through the peptide bond as suggested by Crespi *et al.*[5] Similarly, hydrolysis of a (Leu Cys) chromopeptide under mild conditions liberated no free amino acids, so that in this peptide the bond to PCB is apparently through the sulfur atom and not through a peptide bond. Experiments with various metal ions (Table 5) also support

TABLE 4. AMINO ACID COMPOSITION OF CHROMOPEPTIDES OF FRACTION THREE AND A FRACTION FROM SOLVENT SYSTEM CA*

	Micromoles × 10^3 of PCB or amino acid		
	Expt. 33	Expt. 46 3–2	Expt. 40B
Chromatographic solvents:	AB	A^2C	CA
PCB:	100 (342)	100 (110)	100 (81)
Amino acid			
Aspartic acid	4	15	59
Threonine	—	3	—
Serine	4	11	45
Glutamic acid	3	15	1
Proline	—	—	—
Glycine	—	10	9
Alanine	6	7	5
Cysteine	37	80	54
Valine	—	5	6
Methionine	—	4	2
Isoleucine	—	5	8
Leucine	49	83	59
Tyrosine	—	—	—
Phenylalanine	—	—	—

* See footnotes to Table 1.

the notion of a linkage to sulfur, as the addition of metal ions that react strongly with sulfide lead to a greatly enhanced liberation of PCB. The extent of liberation of PCB by these metals correlates fairly well with the K_{sp} values of the metal sulfides. Those metals with filled or nearly filled *d*-shells, as Hg^{2+}, Ag^{+}, Cd^{2+}, form very insoluble sulfides and are more efficient at liberating PCB than cations such as Fe^{2+}, Mn^{2+}, and Co^{2+} which form more-soluble sulfides.

These data are in agreement with the results of digestion experiments reported earlier by Crespi *et al.*[5] In these earlier experiments, phycocyanin was digested with trypsin, chymotrypsin and carboxypeptidase A. These data indicated a chromopeptide composed of (Ser Gly Ala Asp Leu) and probably Cys, as a similar level of cysteic acid was observed in these experiments. The question of whether or not the cysteic acid peak observed on amino acid analysis is genuine or an artifact has also plagued the experiments reported here, but we have found that if the hydrolysis tubes are especially well degassed, the formation of cysteic acid is

TABLE V. EXTRACTION OF FREE PCB FROM PHYCOCYANIN AFTER TREATMENT WITH METAL IONS

Metal ion	Percent of total PCB extracted	K_{sp} for the metal sulfide*
Hg^{2+}	59	3×10^{-31}
Hg^{+}	27	1×10^{-12}
Ag^{+}	35	3×10^{-14}
Cd^{2+}	32	5×10^{-6}
Pb^{2+}	27	7×10^{-7}
Co^{2+}	12	2×10^{-4}
Fe^{2+}	12	3×10^{-4}
Mn^{2+}	16	6×10^{-1}
None	0·6	—
$HgCl_2$ in MeOH†	88	
MeOH alone	60	
MeOH, acidified	65	

* These values, for an acidic solution, taken from I. M. KOLTOFF and E. B. SANDELL, *Textbook of Quantitative Inorganic Analysis*, p. 68, Macmillan, New York, N.Y. (1952).

† The methanol mixtures were held at 60° for 3 hr before extraction of free PCB.

minimized and cysteine and cystine are observed. Also, the work of Troxler and Lester[15] indicates that during amino acid analysis PCB fragments are not eluted at the cysteic acid elution volume, so the cysteic acid in our experiments may be taken as derived from cysteine. O'Carra *et al.*[16] observed an interaction in strongly acidic media between urobilinoid compounds and thiol compounds. While this observation would appear to have little bearing on these experiments, it does raise the question as to possibility of artifact PCB–peptide interaction, although the data seem much too consistent to be explained in this way. Nevertheless, we have digested various combinations of free PCB, Nagarse, bovine serum albumin, serum globilin, and cysteine, but after chloroform extraction there was never more than 4 per cent of the initial amount of PCB left in the aqueous phase. Consequently, we judge our results real.

In work with peptic digests of the biliprotein phycoerythrin, Fujiwara[17] observed that glutamic acid, aspartic acid, and cysteine were common to all the chromopeptides isolated. She speculated on the possibility of a thio-ether link from apoprotein to bilin. Jones and Fujimori,[18] in studies of the interaction of sulfhydryl reagents and other denaturants with phycoerythrin, conclude that cysteine is important to the integrity of this protein. The data presented here suggest a linkage from PCB to a cysteine side-chain of the protein, perhaps a linkage analogous to that in cytochrome c, but a thio–ether type of bond is not likely to be ruptured by boiling, neutral methanol, conditions that lead to the liberation of PCB. We would suggest that there is also an ester bond involving the carboxyl group of an aspartic acid side-chain and the hydroxyl group of the enol form of ring A of PCB. The methanolysis of phycocyanin could then proceed as outlined in Fig. 1. A transesterification reaction would break the ester link and the PCB would go into the keto form at ring A. This keto group would

[15] R. F. TROXLER and R. LESTER, *Plant Physiol.* **43**, 1737 (1968).
[16] P. O'CARRA, C. O'hEOCHA and D. M. CARROLL, *Biochemistry* **3**, 1343 (1964).
[17] T. FUJIWARA, *J. Biochem. (Japan)* **44**, 723 (1957).
[18] R. F. JONES and E. FUJIMORI, *Physiol. Plantarium* **14**, 253 (1961).

strongly activate position 2 on ring A, causing the elimination of the cysteine side-chain and formation of an ethylidene group. That the keto group can exert a powerful activating effect on the constituents of ring A has been shown by the fact that the hydrogens of the ethylidene methyl group of free PCB are exchangeable in refluxing, neutral methanol.[5] The enhancement effect of metal ions is probably a combination denaturant effect leading to hydrolysis of the ester linkage and an enhanced rupture of the thio–ether bond. Models built with CPK atomic models (Schwarz) indicate that the double mode of attachment of PCB proposed here is sterically possible and suggest that the full sequence about the bonds to the prosthetic group is Gly Asp Ser Leu Cys Ala.

Recent work by Rüdiger and O'Carra[19] involving oxidative degradation of the bilin on the intact protein, or on peptides, indicates that PCB is attached to apoprotein both through the A ring and one of the middle rings. However, there is some difficulty in reconciling attachment to protein through a middle ring, presumably an ester bond involving a propionic acid side-chain of the bilin, with the lack of esterification of these propionic acid side-chains during release of the bilin by hot methanol. Rüdiger and O'Carra[19] suggest that regions of the protein may cooperate in the release of the pigment in hot methanol. One may also argue that under their more mild, acidic oxidizing conditions there may be a cooperative effect with adjacent peptide side-chains leading to the formation of an artifact ester link from an amino acid hydroxyl group to a propionic acid side-chain. The mode of attachment suggested in Fig. 1 has the advantages of being consistent with the action of hot methanol on phycocyanin. Alternately, one may suggest a thio-ether linkage as in Fig. 1 but with an ester link from a propionic acid side-chain to the protein. Additional evidence is needed concerning this second point of attachment.

It is possible that the bonding of PCB to apoprotein suggested here may represent only a portion of the PCB in phycocyanin, but the consistency of the data indicates that all the PCB is bonded in the same way. Since PCB (mol. wt. 586) is present in phycocyanin to the extent of 4·0 per cent[5, 15] one calculates a minimum molecular weight for phycocyanin of 15,000. Other studies with phycocyanin indicate a molecular weight for the phycocyanin monomer of 30,000 or 45,000[20–23] and one cysteine residue per minimum molecular weight unit of 15,000, so the mode of attachment suggested here could easily be true for all PCB molecules in phycocyanin. The observation by Boucher *et al.*[24] that there may be two absorbing species in phycocyanin, one absorbing at ~590 nm and the other at ~620 nm, could be the result of protonation of the pyrrolenine nitrogen of ring A of some of the PCB chromophore, as protonation will cause an increase of about 30 nm in the absorption maximum of PCB.[5, 25]

EXPERIMENTAL

Purification and Proteolysis of Phycocyanin

Phycocyanin was extracted from *Phormidium luridum*[26] and was purified by $(NH_4)_2SO_4$ fractionation and chromatography on ECTEOLA (Mann). Typically, 50 ml of a 2% solution of phycocyanin of purity index 4·5 or greater,[27] in 0·01 M sodium phosphate buffer, pH 7·5, was digested[12] with Nagarse (Enzyme Develop-

[19] W. Rüdiger and P. O'Carra, *European J. Biochem.* **7**, 509 (1969).
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[27] R. M. Rosenberg, H. L. Crespi and J. J. Katz, *Biochim. Biophys. Acta* **175**, 31 (1969).

ment Corp.) for 30–35 min at 32° followed by 18–20 hr at 5°. Half the enzyme was added at zero time and the other half at 15 min. The final concentration of Nagarse was 0·2 per cent. By the end of the digestion period, the pH had fallen to about 6·7.

Extraction Procedures

The digest was taken to pH 4·0 with dil. HCl and extracted vigorously two or three times with $CHCl_3$, the extracts being pooled and dried by rotary evaporation at 30°. The aqueous solution was then extracted twice with equal volumes of butanol.[28] The butanol extracts were pooled and washed twice with distilled water. Emulsions were broken by centrifugation. The chromopeptides were then extracted back into an aqueous phase by extracting the butanol extracts once with distilled water containing a few drops of 0·1 M Na_3PO_4. The blue aqueous phase, pH 7, was dried by rotary evaporation at 35–40° and is referred to below as the "butanol soluble" peptides.

Chromatography and Chromopeptide Analysis

Dried extracts were taken up in methanol or in methanol with 1 per cent (v/v) HOAc and streaked (Rodder Streaker) onto plates coated with Adsorbosil 5 (Anspec) for preparative TLC. Extracts were first fractionated in *n*-BuOH–pyridine–H_2O (3:1:1) (System A), followed by rechromatography with System A or with *n*-BuOH–HOAc–H_2O (4:1:1) (System B), or with toluene–HOAc–H_2O (10:10:1) (System C). The various fractions were eluted with methanol, blown dry under N_2, and finally hydrolyzed with 6 N HCl in evacuated, sealed tubes. Amino acid analyses were done with a Beckman-Spinco 120 Analyser. The amount of PCB in each peptide was estimated from its absorbancy at 600 nm using the absorptivity of free PCB.[5] This procedure will lead to some scatter in the results for PCB content of the peptides, as the true absorptivity of each chromopeptide will deviate from that of free PCB due to residual interactions with still-attached amino acids.

Reaction of Phycocyanin with Metal Ions

The procedure of Paul[29] was used with slight modification. The salt solutions were 0·01 M in metal ion and contained 1 per cent (v/v) HOAc. To 5 ml of each metal salt solution was added 2·6 mg (0·1 ml) of phycocyanin solution. The mixtures were then held at 50° for 90 min, after which free PCB was extracted with $CHCl_3$ and determined spectrophotometrically.

Acknowledgements—We thank Drs. J. J. Katz and W. G. Brown for critical readings of the manuscript and Miss Alyce R. Adams and Mr. Stephen M. Heim for their technical assistance.

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