Affinity of Phycocyanin Chromopeptides to Histidyl-Sepharose Gels: A Model for Histidine-Tetrapyrrol-Interactions in Biliproteins

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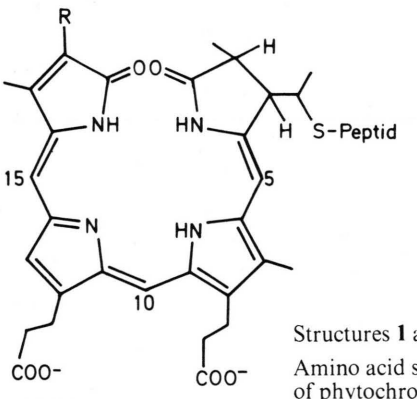
C-Phycocyanin from the cyanobacterium *Spirulina maxima* was digested with pepsin to yield chromopeptides and colorless peptides. This mixture was applied to columns of histidyl-Sepharose under a variety of conditions (pH-value, ionic strength of buffer). We found a good separation of several chromopeptides from each other and from colorless peptides due to differential interaction of phycocyanobilin chromophore with the histidyl residue of the gel. The separation is suppressed by the use of imidazole buffer. Control experiments were performed with purified chromopeptides and with octyl-Sepharose and DEAE-Sepharose. The nature of interaction which probably involves charge transfer interaction besides hydrophobic and ionic forces is discussed with regard to the significance for phytochrome.

Introduction

The functions of plant biliproteins are as diverse as light harvesting for photosynthesis (*e.g.* phycocyanin) and as signal perception for photomorphogenesis (phytochrome). The chemical structure of the light absorbing chromophores, phytochromobilin (**1**) and phycocyanobilin (**2**), are nearly identical [1–4]. The differences in the properties, namely the high photochemical stability of **2** in native phycocyanin and the high photochemical reactivity of **1** in native phytochrome, must be caused by interactions of these chromophores with surrounding amino acids of the polypeptide chain. Inspection of the amino acid sequence reveals the presence of two histidine residues next to the chromophore in phytochrome [4, 5] which are absent from the chromophore regions of phycocyanin [3, 6–8].

If specific interactions between the imidazole moiety of histidine and phytochromobilin exist, such interactions should also be possible between histidine and phycocyanobilin. To test this possibility, we used matrix-linked histidine, namely histidyl-Sepharose gels. Chromatography of a mixture of chromopeptides and colorless peptides obtained by proteolysis of phycocyanin should demonstrate an eventual affinity of the chromophore **2** to this gel:



ZZZ

1: R = CH=CH$_2$

2: R = C$_2$H$_5$

Structures **1** and **2**

Amino acid sequences of phytochrome-peptide [4, 5]

Amino acid sequences of phycocyanin-peptides [6–8]

Phytochrome

Leu-Arg-Ala-Pro-His-Ser-*Cys*-His-Leu-Gln-Tyr

Phycocyanin

α	a)	Gly-Lys-Ser-Lys-*Cys*-Ala-Arg-Asp-Ile-Gly
	b)	Gly-Lys-Ala-Lys-*Cys*-Ala-Arg-Asp
	c)	Gly-Lys-Ala-Lys-*Cys*-Ala-Arg-Asp-Ile-Gly
βI	a)	Thr-Met-Ala-Ala-*Cys*-Leu-Arg-Asp-Met-Glu
	b)	Ala-Ala-*Cys*-Leu-Arg-Asp-Met
	c)	Arg-Met-Ala-Ala-*Cys*-Leu-Arg-Asp-Met-Gln
βII	a)	Thr-Lys-Gly-Asp-*Cys*-Ser-Ala-Leu-Ile-Ser
	b)	Thr-Gln-Gly-Asp-(*Cys*, Ser)-Ala-Ile-Ser-Glu
	c)	Thr-Thr-Gly-Asp-*Cys*-Ser-Ala-Leu-Met-Ala

Fig. 1. Amino acid sequences of the chromophore regions of phytochrome from oats [4, 5] and of phycocyanin from a) *Mastigocladus laminosus* [6]; b) *Synechococcus* 6301 [8]; c) *Cyanidium caldarium* [7]. The chromophore binding amino acid (Cys) is printed in italics.

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the chromopeptide should then have higher retention times than the colorless peptides. This effect and the nature of interactions is investigated in the present paper in detail.

Materials and Methods

Material

The lyophilized preparation of the alga *Spirulina maxima* was a gift of Prof. H. Scheer (München). Pepsin was purchased from SIGMA Chemical Co., the substituted gels of Sepharose, DEAE- and Octyl-Sepharose were commercial products form Pharmacia (Uppsala). The Histidyl-Sepharose 4B gel was prepared according to [9] with DL-histidine. The degree of gel substitution determined by micro Kjeldahl was 0.6 mM/g of dried gel. The columns of diameter 1.0 cm and adjustable volume by piston adapters were a gift from B.M.C., Uppsala. Chemicals and apparatus for electrofocusing were products from LKB. The Silica plates for HPTLC and the other chemical were purchased from Merck (Darmstadt). The absorbance of chromatographic fractions was determined with a HP 8450 A UV-Vis Spectrophotometer (Hewlett Packard).

Preparation of phycocyanin peptides

50 g of lyophilized *Spirulina maxima* algae were suspended in 300 ml 0.002 M Tris-HCL buffer at pH 7.8, then frozen at −20 °C and thawed at ambient (20 °C) temperature. The procedure was repeated three times. The suspension was then centrifugated at 20 000×*g* for 30 min, the supernatant containing C-phycocyanin was lyophilized and kept in the dark at −20 °C until use. For digestion, samples of the lyophilized phycocyanin preparation (10 mg) were dissolved in 100 ml distilled water and acidified with 5 ml formic acid (final pH 1.5 to 2.0). The solution was incubated with 0.5 mg pepsin for 1 h at 37 °C (water bath) and then immediately lyophilized. Samples of this digest (= mixture of chromopeptides and colorless peptides) were dissolved in the desired buffer (see Figs. 2−6).

Purification of single chromopeptides was achieved by chromatography of this digest on Biogel P-10 with a step-gradient 5 to 10% aqueous formic acid, subsequent chromatography on silica gel with water and 20% aqueous pyridine and isoelectric focusing as described by Thümmler and Rüdiger [10]. The two purified fractions used in the present study were those with isoelectric points at pH 3.5 (amino acid analysis in molar ratios: Asx 1.9; Thur 0,5; Ser 0,6; Glx 0,4; Gly 1,5; Ala 1,6; Ile 1,0; Leu 0,7) and pH 4.1 (Asx 1,6; Ser 0,5; Glx 0,9; Gly 1,3; Ala 1,4; Ile 0,4; Lys 1,0; Arg 0,9). The former is derived from β-subunit chromophore II region, the latter from the α-subunit chromophore region [10].

All manipulations of the pigments were carried out in dim light. The chromatography columns were covered with aluminium foil. The buffers were saturated with argon before use. The columns were rinsed before use with a solution of 0.1 M ascorbic acid, 0.005 M EDTA to prevent oxidation and metal complexation of the chromopeptides. The absorption measurements were realized with a circulation cuvette and a spectrophotometer previously programmed in order to record and process the spectra of the eluates at 5 min intervals. The chromatography was performed at 22 °C. The samples were concentrated by lyophilization before thin layer chromatography. The solvent system used was butanol/acetic acid/water 4:4:1 (v:v:v).

Results

Interactions between the phycocyanobilin chromophore and histidine can easily be demonstrated by chromatography of a peptide/chromopeptide mixture on histidyl-Sepharose at pH 6 (Fig. 2): The chromopeptides are more delayed (Ve/Vt = 1.44 and 1.61) than the colorless peptides (Ve/Vt = 1.09). Similar results were obtained without EDTA in the buffer (not shown here) but the separation of the two chromopeptide fractions was not so pronounced in this case. The chromopeptide fractions so purified do not contain colorless peptides. The TLC analysis of these purified chromopeptide fractions revealed that the ninhydrin coloration corresponded only to the chromopeptide fractions. We could not detect any non-chromophoric peptides in these fractions. The observed peaks at Ve/Vt = 1.44 and Ve/Vt = 1.61 correspond to two main chromopeptides of different retention. These two chromopeptides are eluted in the same order on histidyl-Sepharose as on the silica plates.

If the buffer contained 2 M NaCl, there was only little retention of chromopeptides over that of colorless peptides (Table I). The residual retention is probably hydrophobic because at this salt concentra-

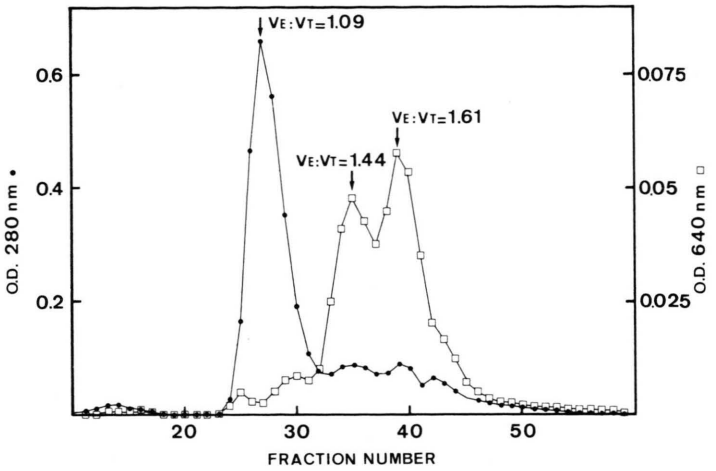

Fig. 2. Elution pattern of phycocyanin digest on Histidyl-Sepharose 4 B column (1 × 22.8 cm), sample: 5 mg dry extract in 0.5 ml buffer, O.D. 640 = 0.84; O.D. 280 = 0.56 × 10, fraction volume 0.77, 0.1 M ammonium acetate 5 mM EDTA buffer at pH 6.0, flow rate 9.23 ml/h. ● Absorbance at 280 nm, □ absorbance at 640 nm.

Table I. Various modifications of conditions from Figs. 2 and 3 suppressing the separation, relative retention of chromopeptides and colorless peptides on histidyl Sepharose 4B and Sepharose 4B.

Condition	Colorless peptides Ve/Vt	Chromopeptides Ve/Vt
Sepharose 4B		
0.1 M ammonium acetate buffer, pH 5.0	0.91	0.91
histidyl Sepharose 4 B 0.1 M ammonium acetate buffer pH 6.0, plus 2 M NaCl	1.04	1.12
histidyl Sepharose 4 B 0.1 M ammonium acetate buffer, pH 4.0	1.15	1.45
histidyl Sepharose 4 B 0.1 M ammonium formiate buffer, pH 3.0	1.06	1.01
histidyl Sepharose 4 B 0.2 M imidazole HCl buffer, pH 5.0	1.10	1.18

tion ionic interactions are cancelled while hydrophobic interactions are increased.

The interaction between chromophore and histidine seems to be even more pronounced at pH 5 (Fig. 3). Whereas the retention of the colorless peptides remains nearly unchanged (Ve/Vt = 1.17 versus 1.09 at pH 6), the chromopeptides are specifically delayed (Ve/Vt = 1.28, 1.78 and 2.38). The elution order of the chromopeptides is not modified but a third, minor chromopeptide fraction is (partially) separated from the main two fractions. Strikingly, when the chromopeptides are eluted in the same conditions but on Sepharose 4B no separation occurs as seen in Table I. This shows that there is no interaction of chromopeptides with the matrix as in the case of Biogel. Moreover, when the elution is effected on histidyl Sepharose at pH 5 but with an imidazole buffer as competitor of histidyl the separation is quite totally suppressed (Table I). This

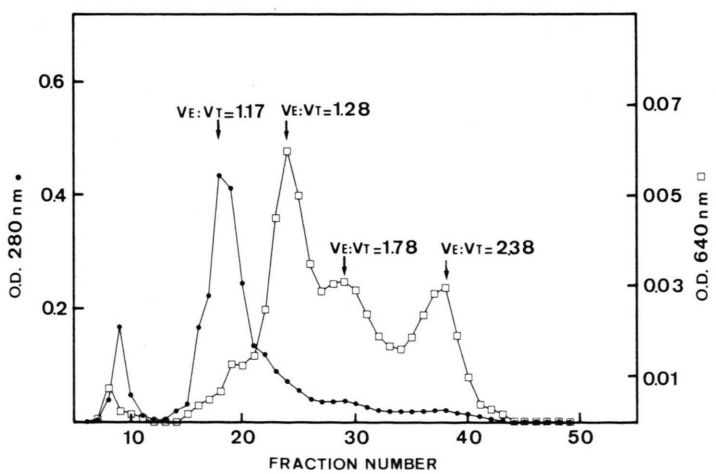

Fig. 3. Elution pattern of phycocyanin digest on Histidyl-Sepharose 4 B column (1 × 15.8 cm), sample: 5 mg dry extract in 0.5 ml buffer, O.D. 640 = 0.75; O.D. 280 = 0.73 × 10, fraction volume 0.83 ml, 0.1 M ammonium acetate buffer at pH 5.0, flow rate 10 ml/h. ● Absorbance at 280 nm, □ absorbance at 640 nm.

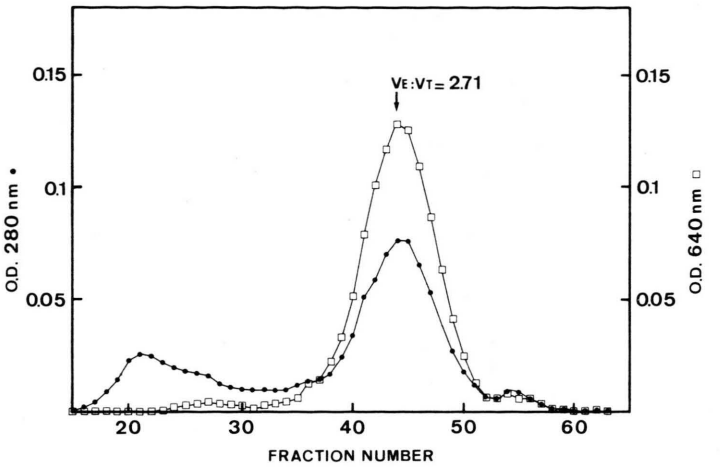

Fig. 4a. Elution pattern of purified phycocyanin chromopeptide (IEP=3.5) [10] on Histidyl-Sepharose 4 B column (1×16.2 cm), sample: 0.5 ml buffer, O.D. 640=2.40; O.D. 280=2.24, fraction volume 0.83, 0.1 M ammonium acetate buffer at pH 5.0, flow rate 10 ml/h. ● Absorbance at 280 nm, □ absorbance at 640 nm.

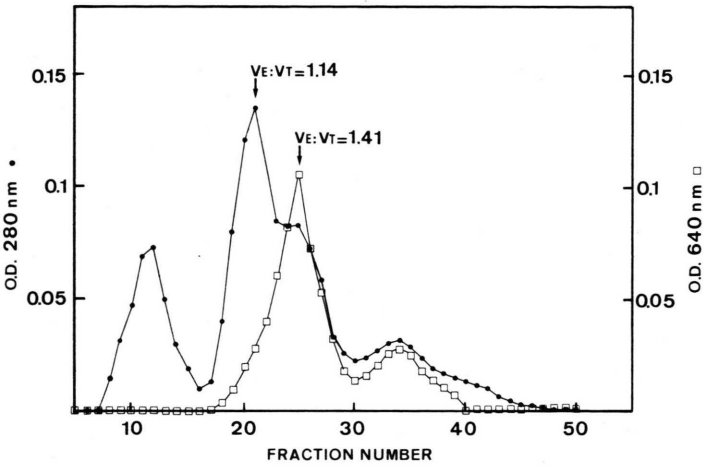

Fig. 4b. Elution pattern of purified phycocyanin chromopeptide (IEP=4.1) [10] on Histidyl-Sepharose 4 B column (1×16.2 cm), sample: 0.5 ml buffer, O.D. 640=0.156×10; O.D. 280=0.138×10, fraction volume 0.83, 0.1 M ammonium acetate buffer at pH 5.0, flow rate 10 ml/h. ● Absorbance at 280 nm, □ absorbance at 640 nm.

demonstrates the specific interactions of the imidazole ring of histidyl ligand with the chromopeptides.

To investigate the role of the chromophore on the one hand and the role of the particular amino acids of the chromopeptides on the other hand in more details, we used purified chromopeptides [10] for the same procedure on histidyl-Sepharose at pH 5 (Fig. 4). The retention times are higher for the purified chromopeptides than for the same chromopeptides mixed with colorless peptides. The chromopeptide derived from the β-subunit chromophor II region (IEP=3.5) has Ve/Vt=2.71 in the pure state and Ve/Vt=2.38 in the mixture with other peptides (Fig. 4a and 3). The chromopeptide derived from the α-subunit chromophore region (IEP=4.1) has Ve/Vt=1.41 in the pure state versus Ve/Vt=1.28 in the mixture (Fig. 4b and 3). The identity of the chromopeptides was checked in each case by thin layer chromatography. It may be that some colorless peptides compete with histidyl-Sepharose in the interaction with the chromophore. It is obvious that histidine-containing peptides would compete in this way. But also peptides containing other basic amino acids could possibly have a similar effect. This can be concluded from our data which demonstrate that the affinity of histidyl-Sepharose to the chromopeptide containing arginine and lysine (Fig. 4) is lower than that to the chromopeptide lacking these amino acids (Fig. 4) although the chromophore is identical in both chromopeptides. Also chain length and amino acid analysis are (apart from Arg and Lys, see above, and one Leu) nearly identical (see

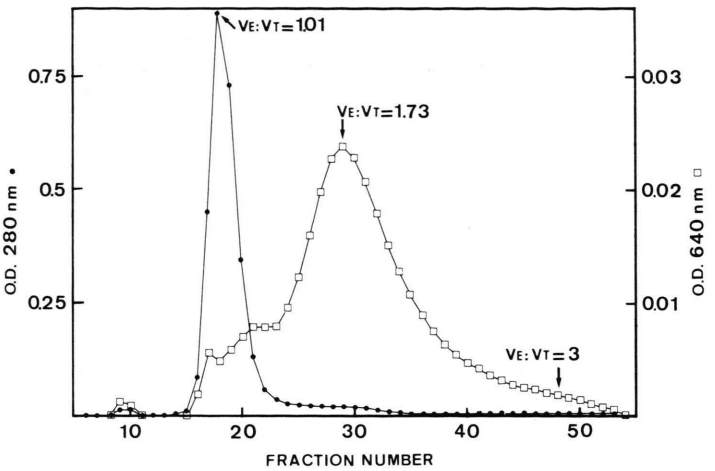

Fig. 5. Elution pattern of phycocyanin digest on Octyl-Sepharose 4 B column (1×16.2), sample: 5 mg dry extract in 0.5 ml buffer, O.D. 640=0.60; O.D. 280=0.559×10, fraction volume 0.83 ml, 0.1 M ammonium acetate buffer at pH 5.0, flow rate 10 ml/h. ● Absorbance at 280 nm, □ absorbance at 640 nm

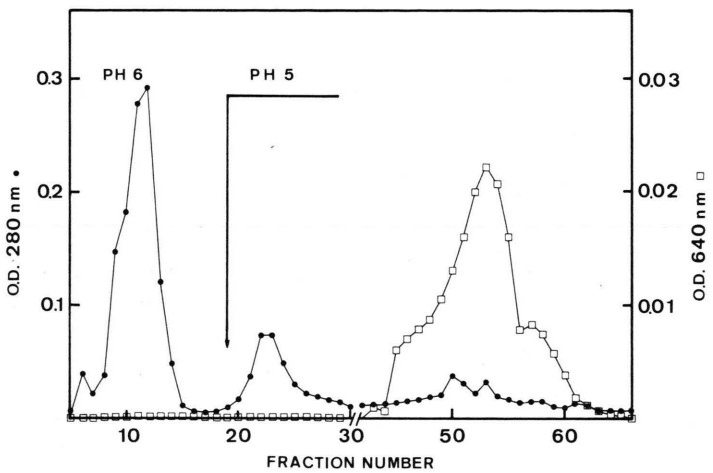

Fig. 6. Elution pattern of phycocyanin digest on DEAE-Sepharose 6 B column (1×8.7 cm), sample: 5 mg dry extract in 0.5 ml buffer, O.D. 640=0.64; O.D. 280=0.58×10, fraction volume 0.83 ml, 0.1 M ammonium acetate buffer at pH 6.0 then 0.1 M ammonium acetate buffer at pH 5.0, flow rate 10 ml/h. ● Absorbance at 280 nm, □ absorbance at 640 nm.

Materials and Methods). But it cannot be excluded at this time that differences in the amino acid sequence contribute to the differencee in the retention of both chromopeptides.

The separation optimum for chromopeptides seems to be at about pH 5. There is no separation at pH 4. Colorless peptides and chromopeptides are equally delayed. The elution profile shows a spreading of the peaks and poor resolution.

There is also no separation between the chromopeptides and peptidic peaks at pH 3 which is lower than the isoelectric point of chromopeptides. Chromopeptides and peptides show no more attraction for histidyl-Sepharose. The repulsion of histidyl by the amino acid residues in their cationic form becomes preponderant.

To check whether hydrophobic interactions can contribute to separation at pH 5, we also performed the chromatography on octyl-Sepharose gel. Most of the chromopeptides are much delayed, showing a peak maximum Ve/Vt=1.73 and an important tailing till Ve/Vt=3. A less delayed minor fraction forms a shoulder at Ve/Vt=1.21 (Fig. 5). The whole chromopeptides are retained on the column at 2 M NaCl and a part of the colorless peptides as well. The relative retention volume is over 10 (Ve/Vt). These fractions are quickly recovered by changing the buffer to 0 M NaCl. When elution is effected at 1 M NaCl chromopeptides are still much delayed. Several small chromopeptidic fractions are separated with Ve/Vt=0.886, 1.343, 2.191, 2.648 and over. However, there is no good resolution because the

peaks are flattened and the fractions much diluted. The hydrophobic interaction is important but octyl-Sepharose ligand does not separate all the chromopeptides from the eolorless peptide fraction and no specificity for sinlge chromopeptides could be observed by TLC analysis.

The ionic character of chromopeptide-matrix interactions was then tested by elution from the ion exchanger DEAE-Sepharose (Fig. 6). We obtained a sharp separation between peptides and chromopeptides, but there was no difference of retention of the various chromopeptides. The chromopeptides were much delayed on the column while the colorless peptides were quickly eluted out of the column (Ve/Vt = 1.16). After lowering the pH of the buffer from 6 to 5, another colorless peptidic fraction is eluted. The chromopeptides were much delayed. They were eluted after percolating 3.8 time the volume of the column by pH 5 buffer. However, there was no good resolution between the different chromopeptidic fractions. At pH 4, or 3, or at pH 3 and 1 M NaCl, neither colorless peptides nor chromopeptides were desorbed. This seems to the typical for the ionic effect for the interaction between chromopeptides and DEAE-Sepharose. However, another kind of interaction cannot be excluded because of the structure of DEAE-Sepharose.

Discussion

Chromopeptides from phycocyanin show specific interactions with histidyl-Sepharose. The interaction optimum is found at pH 5. Less interaction is observed at pH 3 – 4, somewhat less also at pH 6. Higher pH values could not be checked because of the instability of chromopeptides at high pH values. The specificity of the interaction leads to separation of different chromopeptides which all contain the same tetrapyrrole chromophore **1a** but different amino acids.

The nature of interaction(s) was investigated by comparison of histidyl-Sepharose with octyl-Sepharose and DEAE-Sepharose. It can be shown that the phycocyanobilin chromophore can form both hydrophobic interactions (with octyl-Sepharose) and ionic interactions (with DEAE-Sepharose). However, both of these types of interactions are different from the observed interactions with histidyl-Sepharose: a) the pH-dependency is quite different, b) no specificity is found with regard to separation of sinlge chromopeptides from each other. Although some hydrophobic and some ionic interaction can be present between chromophore and histidyl-Sepharose, there must be something more specific in addition. This ist clearly demonstrated by the cancellation of histidyl chromopeptide interactions by the imidazole buffer. The pH optimum for this interaction (pH 5 – 6) is very close to the pK-values of both the chromophore (pK = 5.8) and histidine (pK = 6.5). This could allow proton exchange and charge transfer interactions. Such interactions would be very sensitive towards small changes of the pK-value which could be induced *e.g.* by the vicinity of positively charged arginine and lysine or by the particular position of dipole groups (*e.g.* of serine). In accordance with this view, slight differences in the chromophore absorption are found for the various chromopeptides.

It can be expected from these results that the interaction of the chromophores **1a** and **1b** with histidine is stronger than that with other amino acids. This should be relevant especially for phytochrome in which two histidines are in the direct vicinity of the chromophore (Fig. 1). There are two sets of recent data which point to the possibility that such interactions could play a role in the $E \rightarrow Z$ isomerization of the chromophore which is the basis for the $P_{fr} \rightarrow P_r$ transformation [10, 11]: 1. The dark reversion of the *E*- to the *Z*-conformation at higher pH values is much faster in phytochrome peptides than in phycocyanin peptides (Thümmler and Rüdiger, unpublished results). This could be due to catalysis of the $E \rightarrow Z$ transformation by the histidine residues. 2. The high resolution NMR spectrum shows a shift of the histidine proton signals upon phototransformation of the P_{fr} chromopeptide (chromophore with *E*-configuration) to the P_r chromopeptide (chromophore with *Z*-configuration) [Thümmler, Rüdiger, Cmiel and Schneider, unpublished results]. This means that the interaction of the histidines with the P_{fr} chromophore is different from that with the P_r chromophore.

For practical purpose, our results show that a good separation of phycocyanobilinpeptides from colorless peptides can be achieved with DEAE-Sepharose as well as with histidyl-Sepharose. The latter gel material has, however, the advantage that single chromopeptides can be separated from each other in the same procedure which is not the case with other gel materials.

Acknowledgement

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