3: (a) 1% Ethanol–H_2O. Absorbance was measured at 272 nm where ϵ for lactone and hydroxy acid monoanion are 470 and 1830, respectively. Starting with lactone at pH 6.92 and 7.22 gave $K_{eq}' = 4.3$ and 2.0, respectively. Starting with hydroxy acid at pH 6.95 and 7.28 gave $K_{eq}' = 3.9$ and 1.9, respectively. (b) 20% Dioxane–H_2O. Absorbance was measured at 278 nm where ϵ for lactone and hydroxy acid are 74 and 1750, respectively. Starting with hydroxy acid at pH 8.35, 8.51, 8.69, and 8.90 gave $K_{eq}' = 0.63, 0.40, 0.29,$ and 0.17, respectively.

4: Absorbance was measured at 286 nm, where ϵ for lactone, monoanion, and dianion are 20, 1390 and 2300, respectively. Starting with lactone at pH 10.99, 11.40, 11.42, and 11.97 gave K_{eq}' values of 3.4, 1.4, 1.4, and 0.30, respectively. The pK_a value of 5.78 (carboxyl group) estimated from the pH–rate profile for the lactonization of **4** was used to calculate the value of the pH-independent equilibrium constant.

5: Although the mole fraction of dihydrocoumarin present in equilibrium with **5** is very small, the large difference in molar extinction coefficient between lactone ($\epsilon_{240} = 2140$) and hydroxy acid ($\epsilon_{240} = 100$) allows a reasonably accurate measurement of K_{eq}. The absorbance increased from 0.56 to 0.65, yielding $K_{eq} = 8.2 \times 10^{-3}$, starting with **5** at 5.6×10^{-3} M (0.1 M HCl). A second experiment with **5** at 1.15×10^{-2} M gave an absorbance change from 1.154 to 1.350, from which $K_{eq} = 8.3 \times 10^{-3}$ was obtained.

Chromopeptides from Phytochrome. The Structure and Linkage of the P_R Form of the Phytochrome Chromophore

J. Clark Lagarias and Henry Rapoport*

Contribution from the Department of Chemistry and Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. Received December 24, 1979

Abstract: The isolation and chromatographic purification of chromophore-containing peptides from the P_R form of phytochrome treated with pepsin and thermolysin are described. From the amino acid sequence and ^{1}H NMR spectral analysis of phytochromobiliundecapeptide (**2**), the structure of the P_R phytochrome chromophore and the nature of the thioether linkage joining pigment to peptide have been established. Confirmatory evidence was obtained from similar analysis of phytochromobilioctapeptide (**3**). The implications of this structural assignment with respect to the mechanism of the P_R to P_{FR} phototransformation are considered.

Owing to the wide range of light-controlled development and metabolic processes in green plants believed to be mediated by phytochrome, this biliprotein has been exhaustively studied by plant physiologists for many years.[1] Phytochrome has also received extensive study by physical and biological chemists because it exists in two spectrally distinct forms P_R (λ_{max} 665 nm) and P_{FR} (λ_{max} 720 nm) which are interconvertible upon absorption of light.[2] Despite the tremendous interest in this unusual photoreceptor, neither the chemical structure nor the precise nature of the chromophore–protein linkage of the P_R or P_{FR} chromophore has been definitively established.[2]

The numerous structures proposed for the phytochrome chromophore have been based primarily on degradative approaches which have involved spectroscopic analyses of altered forms of the chromophore, released from phytochrome after treatment with refluxing methanol[3] or with chromic acid.[4] In contrast to these previous studies of the phytochrome chromophore, our approach is based on the chromophore as well as the chromophore–protein linkage remaining unchanged throughout the analysis. Previously

we have successfully applied this methodology to the structure elucidation of the β_1-phycocyanobiliheptapeptide (**1**) isolated from *C*-phycocyanin.[5] Now we provide ^{1}H NMR spectroscopic evidence for the structure and linkage of the P_R form of the phytochrome chromophore.

In 1971 Fry and Mumford partially determined the amino acid sequence of a phytochromobiliundecapeptide isolated from "small" oat phytochrome treated with pepsin.[6] In the present investigation, we describe the isolation of phytochromobiliundecapeptide (**2**) and phytochromobilioctapeptide (**3**) following the sequential pepsin–thermolysin digestion of oat phytochrome in the P_R form. ^{1}H NMR spectra were obtained, and their analyses provided proof of the structure and thioether linkage of the P_R form of the phytochrome chromophore.

Results and Discussion

Phytochrome Purification. The routine isolation of 50–60 mg of brushite-purified oat phytochrome with a specific absorption ratio (SAR = A_{667nm}/A_{280nm}) of 0.07[2a] from 4 kg-batches of etiolated oat seedlings was accomplished as described.[7] Crude phytochrome fractions eluted from brushite chromatography were assayed by measuring the double-difference spectra with a modified Cary 118 spectrometer.[8] As shown in Figure 1, this low-purity phytochrome was phototransformable, although a dramatic increase in turbidity accompanied the P_R to P_{FR} conversion.

(1) Phytochrome-mediated physiological responses in plants have been reviewed by: Satter, R. L.; Galston, A. W. In "Chemistry and Biochemistry of Plant Pigments"; Goodwin, T. W., Ed.; Academic Press: New York, 1976, Vol. 1, pp 680–735.

(2) For recent reviews of phytochrome biochemistry and chromophore chemistry see: (a) Pratt, L. H. *Photochem. Photobiol.* **1978**, *27*, 81–105. (b) Kendrick, R. E.; Spruit, C. J. P. *Ibid.* **1977**, *26*, 201–14.

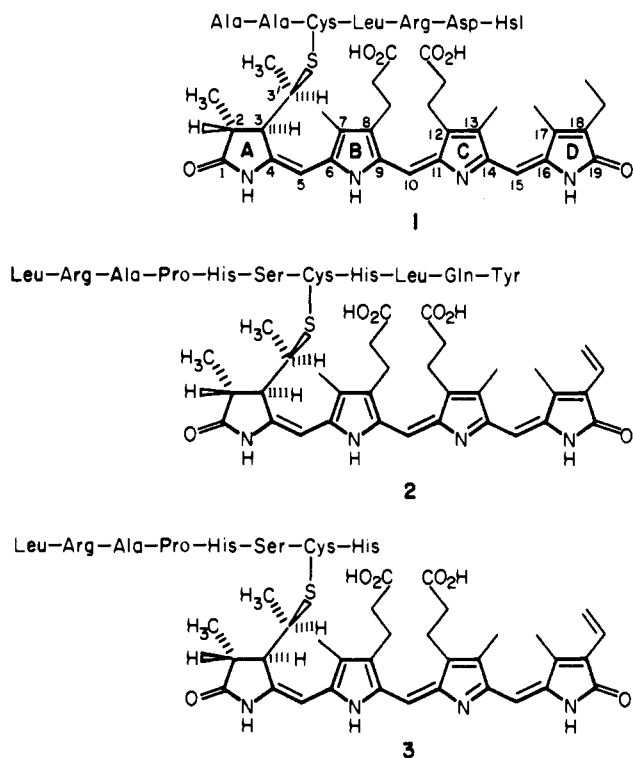(3) Siegelman, H. W.; Turner, B. C.; Hendricks, S. B. *Plant Physiol.* **1966**, *41*, 1289.

(4) (a) Rudiger, W.; Correll, D. L. *Justus Liebigs Ann. Chem.* **1969**, *723*, 208. (b) Rudiger, W. In "Phytochrome"; Mitrakos, K., Shropshire, W., Eds.; Academic Press: New York, 1972; pp 129–41. (c) Grombein, S.; Rudiger, W.; Zimmermann, H. *Z. Physiol. Chem.* **1975**, *356*, 1709. (d) Schoch, S.; Rudiger, W. *Justus Liebigs Ann. Chem.* **1976**, 559. (e) Klein, G.; Grombein, S.; Rudiger, W. *Z. Physiol. Chem.* **1977**, *358*, 1077.

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(6) Fry, K. T.; Mumford, F. E. *Biochem. Biophys. Res. Commun.* **1971**, *45*, 1466.

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(8) Jung, J.; Song, P.-S. *Photochem. Photobiol.* **1979**, *29*, 419.

Ala—Ala—Cys—Leu—Arg—Asp—Hsl

1

Leu—Arg—Ala—Pro—His—Ser—Cys—His—Leu—Gln—Tyr

2

Leu—Arg—Ala—Pro—His—Ser—Cys—His

3

Figure 1. Absorption spectrum of brushite-prepared phytochrome used in this study ($c = 7.33 \times 10^{-6}$ M in 0.1 M K_2HPO_4/KH_2PO_4, pH 7.8, SAR = 0.07): (—) P_R after irradiation at 720 nm; (- - -) P_{FR} after irradiation at 660 nm.

Pepsin–Thermolysin Digestion of Phytochrome. The isolation of a chromopeptide fragment from the pepsin digest of brushite-purified phytochrome in the P_R form was accomplished according to the reported procedure[6] with several modifications. First, the phytochrome used for this study was large, crude phytochrome (SAR = 0.07) instead of small, purified phytochrome (SAR = 0.2–0.9) employed in the previous study.[6,9] A second modification was the pretreatment of the BioGel P4 column with a mixture of 0.1 N ascorbic acid and 0.01 N EDTA. Unless this precaution was taken before application of the digest mixture, a decomposition product (λ_{max} 415 nm) appeared on the column at the expense of compounds with longer wavelength absorptions. During chromatography the majority of the blue color remained attached to the BioGel P4. Even after three column volumes of 1.3 M formic acid, these chromopeptides were not removed from the column. Step elution with 25% aqueous acetic acid proved necessary to desorb the blue peptides from the column. With these modifications, a chromopeptide with spectral characteristics similar to that reported[6] was obtained in 58% yield. This yield was based on absorbance, where the extinction coefficient for P_R at 665 nm was 7.0×10^4 L mol^{-1} cm^{-1} at pH 7.8[10] and for phytochromobilipeptides at 658 nm was 3.2×10^4 L mol^{-1} cm^{-1} in 5% aqueous formic acid.[6]

As shown in Table I, the amino acid composition of our chromopeptide fraction is similar to that reported.[6] The presence of cysteine in this chromopeptide fraction was clearly established.[11] However, the presence of small amounts of threonine, glycine, isoleucine, and phenylalanine, in addition to the low value of arginine, indicated the inhomogeneity of this chromopeptide fraction. The ^1H NMR spectrum of this fraction also showed the presence of a contaminant which appeared to be derived from the polyacrylamide matrix of the BioGel P4 column (see supplementary material, Figure 1).

Table I. Amino Acid Analyses

amino acid	pepsin digest (BioGel P4) chromopeptide fraction,[a] nmol	thermolysin digest HPLC fraction,[b] nmol			
		2	3	4	5
His	21	54	121	79	102 (4)
Arg	5 (8)	19	62 (55)	36	46 (43)
Cya[c]	(13)		(57)		(42)
Asp	4 (7)	4	<1 (<2)	5	
Thr	1 (2)	tr		tr	
Ser	14 (20)	29	47 (54)	44	36 (47)
Glu	14 (17)	23	18 (21)	34	46 (47)
Pro	tr[c]	31	63 (62)	39	52 (48)
Gly	3 (7)	7	14 (16)	9	(2)
Ala	14 (16)	40	67 (74)	46	49 (56)
$^1/_2$Cys		7		11	6
Val	1 (4)	tr	(<1)		
Met		2		tr	
Ile	4 (<2)	5	(3)	<2	
Leu	26 (35)	53	66 (71)	60	109 (100)
Tyr	16	17	14	26	41
Phe	4	6		tr	

[a] Hydrolyzed 27 nmol (based on ϵ_{665} 3.2×10^4 L mol^{-1} cm^{-1}) in 6 N HCl plus 50 μL of 5% phenol at 110 °C for 20 h. Values in parentheses are amino acid yields after 20 h, 110 °C hydrolysis in 6 N HCl plus 30 μL of Me$_2$SO.[11] [b] Hydrolysis as in a: fraction 2, 41 nmol; fraction 3, 68 nmol; fraction 4, 48 nmol; fraction 5, 55 nmol. [c] Abbreviations: tr = trace, Cya = cysteic acid.

Table II. Amino Acid Analysis of Phytochromobiliundecapeptide[a] and Recovery of PTH Amino Acid Derivatives at Each Step of the Edman Degradation

amino acid	original anal.[b]	PTH deriv[c] recovd after each step of the Edman degradation										
		1	2	3	4	5	6	7	8	9	10	11
His	2.0					+			+			
Arg	0.9	+										
Cya[d]	0.9							+				
Ser	0.7						+					
Gln	0.9										+	
Pro	1.0				+							
Ala	1.0			+								
Leu	2.1	+								+		
Tyr	0.8											+

[a] **2**, high-performance liquid chromatography fraction 5, Figure 3. [b] Results from Table I. Relative residue amounts of each amino acid based on Ala = 1.0 residue. [c] PTH derivatives identified by TLC, high-performance liquid chromatography, and mass spectrometry. [d] Cysteic acid determination by the method of ref 11.

The similarity of the amino acid composition of this pepsin chromopeptide fraction with that of the undecapeptide reported

(9) Large losses of phytochrome, which occur at each step of the various published purification procedures,[2] can be avoided by using brushite-purified photochrome. Previously the lack of sufficient material has prevented this type of spectroscopic analysis of chromopeptides derived from photochrome. The use of brushite-prepared phytochrome, which can be obtained rapidly in quantity, has greatly reduced the amount of material and labor necessary for this undertaking.

(10) Tobin, E. M.; Briggs, W. R. *Photochem. Photobiol.* **1973**, *18*, 487.

(11) Glazer, A. N.; Hixson, C. S.; DeLange, R. J. *Anal. Biochem.* **1979**, *92*, 489.
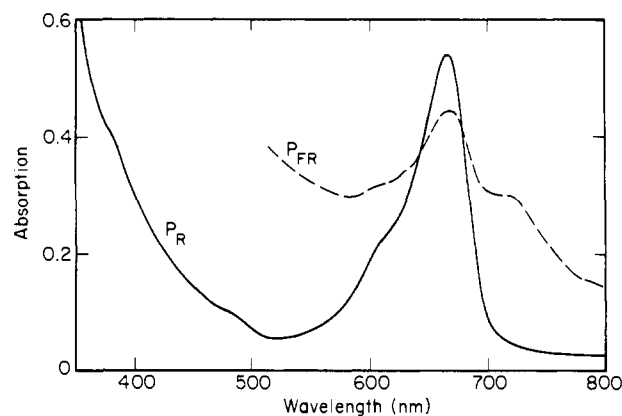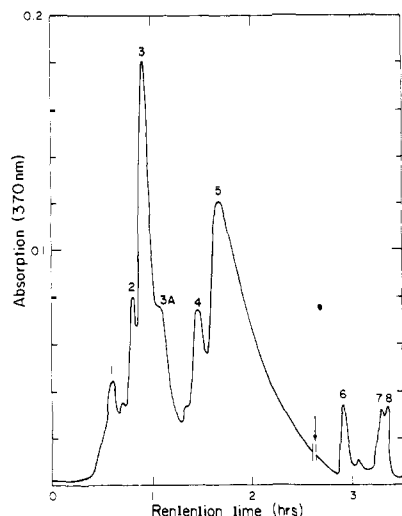
Figure 2. High-performance liquid chromatography of the pepsin chromopeptide fraction after thermolysin digestion. The column contained LiChroprep RP18, 25–40 μm, and was 15 × 500 mm, the mobile phase consisted of 30% CH$_3$CN/70% 0.01 N aqueous trifluoroacetic acid (changed to 44% CH$_3$CN/56% 0.01 N TFA at the arrow), the flow rate was 3 mL/min, and the injection volume was 1.5 mL.
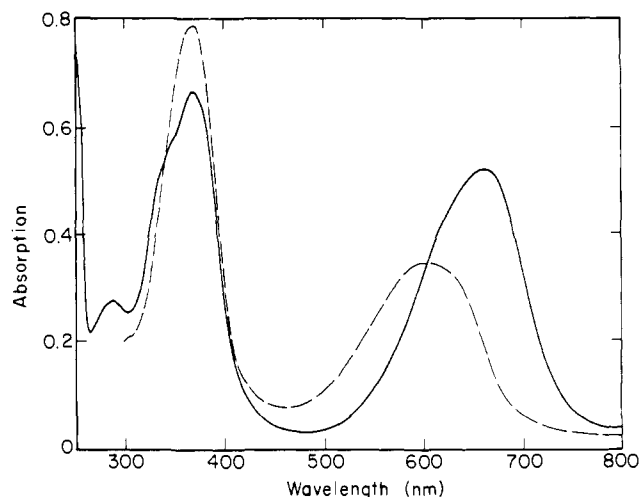
Figure 3. Absorption spectrum of phytochromobiliundecapeptide (**2**, high-performance liquid chromatography fraction 5): (—) 0.01 N trifluoroacetic acid, $c = 1.72 \times 10^{-5}$ M; (- - -) pyridine, $c = 2.15 \times 10^{-5}$ M.
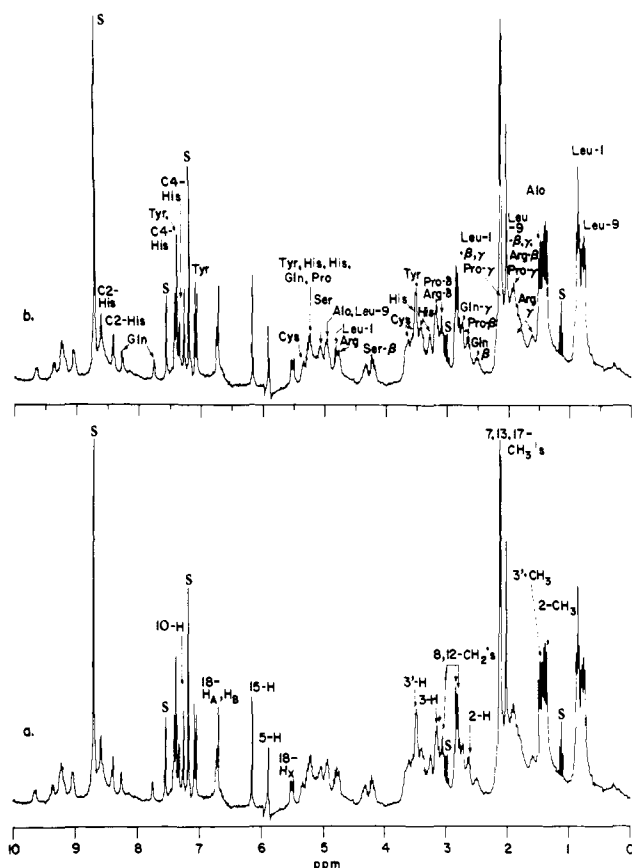
previously[6] suggested that a thermolysin cleavage would result in shortening of the peptide chain. Thermolysin has been an effective tool for sequence analysis of chromopeptides from *C*-phycocyanin, through selective cleavage at the amino termini of leucine or isoleucine residues.[12] Thus we subjected our pepsin chromopeptide fraction to thermolysin digestion with the following modification of the reported procedure. Before incubation at 37 °C for 4 h, the initial chromopeptide mixture was dissolved in 0.1 N ammonium bicarbonate with the addition of 3% thermolysin (w/w), degassed by freeze–thawing, and sealed under vacuum. Taking these precautions and avoiding exposure of the sample to light virtually eliminated the color changes which accompany chromophore decomposition. After digestion, the mixture was applied to a Sephadex G50 column equilibrated with 25% acetic acid. Elution with 25% acetic acid afforded a 47% overall recovery of a chromopeptide fraction with unchanged absorption properties (see supplementary material, Figure 2).

Subsequent high-performance liquid chromatography[13] of this fraction resolved five major chromopeptide components (Figure 2) with indistinguishable absorption spectra in 30% overall yield. The amino acid compositions of fractions 2 through 5 were next determined (Table I). These results showed that the four fractions were different-sized peptides derived from the same polypeptide chain. The peptide obtained in the largest quantity and the purest, fraction 5, was an undecapeptide **2** with the same amino acid composition as the reported phytochromopeptide.[6] The absorption spectrum of the undecapeptide **2** is shown in Figure 3.

Fraction 3 was an octapeptide **3** with an amino acid composition identical with that of the undecapeptide less the three residues leucine, glutamine, and tyrosine. Although fractions 2 and 4 were not as pure as the octa- and undecapeptides, these fractions appear to be a heptapeptide and a decapeptide, respectively. The similarity in the core composition of all four chromopeptides showed that these peptides are all derived from the same region of the phytochrome polypeptide chain.[14] Although conventional hydrolysis of fractions 3 and 5 gave low yields of half-cystine, oxidative hydrolysis for 20 h with 0.2 M Me$_2$SO/6 N HCl released 0.8 residue of cysteic acid.[11] This result confirmed the presence

(12) Williams, V. P.; Glazer, A. N. *J. Biol. Chem.* **1978**, *253*, 202.

(13) As described in ref 5, an all-glass and Teflon high-performance liquid chromatography system was used due to the instability of these chromopeptides when exposed to metals.

(14) This result supports the prediction that there is a single bilin chromophore per monomer chain of phytochrome (M_r 120 000). The proof of this hypothesis requires a complete structural analysis of immunoaffinity-purified phytochrome[7] like that described for the analyses of algal biliproteins.[12]

Figure 4. 270-MHz ^{1}H NMR spectrum of phytochromobiliundecapeptide (**2**) in [^{2}H$_5$]pyridine at 25 °C: (a) chromophore assignments; (b) peptide assignments.

of one residue of cysteine in these chromopeptides.

Sequence analysis of the phytochromobiliundecapeptide (**2**) established the amino acid sequence to be Leu-Arg-Ala-Pro-His-Ser-Cys-His-Leu-Gln-Tyr. The results of the Edman degradation of **2** are summarized in Table II. With the exception of the cysteine derivative, the PTH derivatives of the cleaved amino acids were determined by TLC, high-performance liquid chromatography, and mass spectrometry. Back-hydrolysis of the sixth step yielded alanine as a confirmation for the initial presence of serine at this step. After the seventh step of the Edman degradation the blue color, which mostly remained in the sequenator cup throughout the analysis, was extracted into the butyl chloride washes. That cysteine was removed during this step was confirmed

Table III. 270-MHz ^{1}H NMR Assignments for the Bilin Moieties of Phytochromobiliundecapeptide (2) and β_1-Phycocyanobiliheptapeptide $(1)^a$ in $[^2H_5]$Pyridine at 23 °C

chem shift^b			multiplicity and	
2	1	no. of H's	*J*, Hz, for 2	assignt
	1.23	3	t, 7.3	18-CH$_2$CH$_3$
1.38	1.39	3	d, 7.3	2-CH$_3$
1.43	1.48	3	d, 7.3	3'-CH$_3$
2.03	2.02	3	s	7-CH$_3$
2.11	2.07	3	s	17-CH$_3$
2.13	2.12	3	s	13-CH$_3$
	2.48	2	q, 7.3	18-CH$_2$CH$_3$
2.64	2.70	1	dd, 5.0, 7.3	2-H
2.81	2.83	2	t, 7.2 ⎫	
2.84	2.85	2	t, 7.2 ⎬	8,12-CH$_2$CH$_2$COOH
3.09	3.09	2	t, 7.2 ⎫	
3.17	3.17	2	t, 7.2 ⎬	8,12-CH$_2$CH$_2$COOH
3.18	3.15		*c*	3-H
3.50	3.52		*c*	3'-H
5.51		3	dd, 2, 12 (ABX)	18-H$_X$ (vinyl)
5.90	5.87	<1^d	s	5-H
6.16	6.08	1	s	15-H
6.71		1	m, 2, 18 (ABX)	18-H$_B$ (vinyl)
6.73		1	m, 12, 18 (ABX)	18-H$_A$ (vinyl)
7.26	7.29	1	s	10-H

^{a} Assignments from ref 5. ^{b} The chemical shift values are in parts per million from Me$_4$Si and were determined from a residual proton of pyridine (7.81 ppm from Me$_4$Si at 23 °C). ^{c} Overlapping resonances attributed to the peptide obscured these signals. ^{d} The low integral for the C-5 proton appears to be due to deuterium exchange as was observed for 1;^{5} before ^{1}H NMR spectroscopy undecapeptide 2 was exchanged in D$_2$O.

by hydrolysis of the evaporated butyl chloride extracts with 0.2 M Me$_2$SO in 6 N HCl for 20 h. Amino acid analysis of this hydrolysates showed a good recovery of cysteic acid.

^{1}H NMR Spectral Analysis. The 270-MHz ^{1}H NMR spectrum of phytochromobiliundecapeptide (2) in [^{2}H$_5$]pyridine^{15} is shown in Figure 4. Complete analysis of this spectrum shows that with respect to the chromophore moiety this chromopeptide is quite similar to β_1-phycocyanobiliheptapeptide (1).^{5,16}

The major difference between the two spectra can be explained by the replacement of the ethyl group of 1 with a vinyl group as in structure 2. A new ABX pattern in the spectrum of undecapeptide 2 has replaced the high-field A$_3$X$_2$ pattern of the C-18 ethyl group of 1. The assignments of these terminal olefin resonances of 2 shown in Table III were confirmed by computer simulation. As illustrated in Figure 5, excellent agreement between the experimental spectrum of the vinylic ABX pattern and the computer-simulated spectrum was observed. Furthermore, the small differences (<0.1 ppm) between the spectra of the bilin moieties of 1 and 2 (i.e., the lower field values of the C-15 methine bridge hydrogen and the C-17 methyl group) can be attributed to the effect of the vinyl group in 2 on the electron density, especially in ring D.

The assignment of the structure of the dihydro A ring and the 3'-thioether linkage of 2 was based on double-irradiation experiments illustrated in Figure 6. By analogy to the assignments for 1,^{5} the two doublets at 1.38 and 1.43 ppm in the spectrum of chromopeptide 2 have been assigned to the C-2 and C-3' methyl groups. Collapse of the multiplet at 2.64 ppm to a doublet with 5.0-Hz spacing, after irradiation of the C-2 methyl doublet at 1.38 ppm (insert c, Figure 6), supported the dihydro A-ring structure for 2. Irradiation of the 2.64-ppm multiplet led to changes in the multiplicity of the signal at 3.18 ppm (insert b, Figure 6) while also collapsing the 1.38-ppm doublet to a singlet (insert f, Figure 6). The assignment of the C-3 H resonance to 3.18 ppm was

(15) ^{1}H NMR spectra of the phytochromobilipeptides were also recorded in D$_2$O solutions. Aggregation of these peptides, as observed for 1,^{5} made these spectra difficult to interpret. For this reason, the D$_2$O spectral data will not be dealt with in this report.

(16) The assignment of the ^{1}H NMR spectrum of the peptide moiety is included in Table I of the supplementary material.
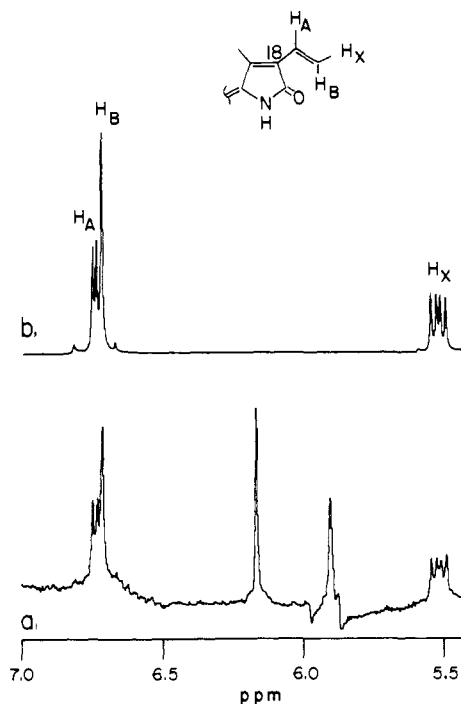

Figure 5. Low-field region of the 270-MHz ^{1}H NMR spectrum of 2: (a) experimentally observed spectrum; (b) computer simulation of the 18-vinyl ABX pattern with J_{AB} = 18 Hz, J_{AX} = 12 Hz, and J_{BX} = 2 Hz (line width assumed in spectrum simulation, 2.0 Hz).
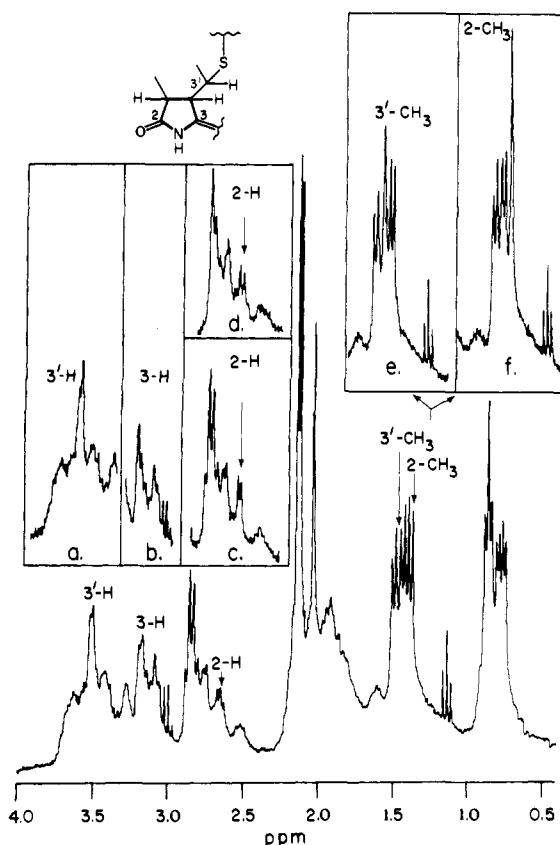

Figure 6. High-field region of the 270-MHz ^{1}H NMR spectrum of 2. Inserts show spectra after double-irradiation experiments: (a) irradiation of doublet at 1.43 ppm; (b and f) irradiation of multiplet at 2.64 ppm; (c) irradiation of doublet at 1.38 ppm; (d) irradiation of multiplet at 3.18 ppm; (e) irradiation of multiplet at 3.50 ppm.

confirmed when the C-2 proton multiplet at 2.64 ppm became a quartet with *J* = 7.3 Hz during double irradiation at 3.18 ppm (insert d, Figure 6). The vicinal relationship of the C-3' proton

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at 3.50 ppm and the C-3' methyl at 1.44 ppm was similarly established by spin-decoupling experiments (inserts a and e, Figure 6).

A second linkage involving the propionic acid side chains of **2** is highly unlikely due to the near identity of the chemical shifts of the propionic acid methylenes (C-8 and C-12) of the two chromopeptides **1** and **2**. An ester linkage through the tyrosine phenolic group can be clearly ruled out because of the isolation of chromopeptides lacking tyrosine. The ^{1}H NMR spectrum of one of these peptides, phytochromobilioctapeptide (**3**), was obtained and showed no difference in the chemical shifts of the C-8 and C-12 methylenes (see supplementary material, Figure 3). With respect to the thioether linkage in ring A, the spectra of the two phytochromobilipeptides **2** and **3** were the same as well. Furthermore, by comparison of the ^{1}H NMR spectrum of **2** with **1** and other chromopeptides from C-phycocyanin and R-phycoerythrin, bilin–peptide linkages through the side chains of arginine, serine, and glutamine can be ruled out.[17]

Since it is improbable that an ester or amide linkage would be cleaved during the proteolytic digests and subsequent purification procedures used in this study, we conclude that P_R phytochrome is singly bound to the apoprotein through a thioether linkage. Owing to the near identity of the ^{1}H NMR spectra of the two chromopeptides **1** and **2** with respect to the bilin moieties, we have assigned this linkage through ring A of the phytochromobilin in **2** in preference to a ring-D linkage. Experiments to provide direct proof for this assignment are in progress.

Stereochemistry. The similarity of the ^{1}H NMR spectra of the bilin moieties of phytochromobiliundecapeptide (**2**) and β_1-phycocyanobiliheptapeptide (**1**)[5] suggests that the dihydro A ring of both bilins has the trans stereochemistry.[18] The relative stereochemistry at C-3,C-3' was proposed to be *R,R* (or *S,S*) based on the isolation of (*E*)-2-ethylidene-3-methylsuccinimide (**4**) from chromic acid treated phytochrome.[4e] Since none of the *Z* isomer was detected after this treatment, a concerted trans-periplanar elimination of the cysteine thioether linkage was proposed.[4e] Assuming this mechanism, the isolation of the *E* isomer **4** therefore required the stereochemistry at C-3,C-3' to be *R,R* (or *S,S*).[4e] On the basis of these assumptions, the stereochemistry of the dihydro A ring of phytochromobilin (C-2, C-3, C-3') is *R,R,R* (or *S,S,S*). The *R,R,R* representation has been incorporated into structures **1**, **2**, and **3**. Since the optical activity of *E*-succinimide **4** has not yet been reported, the absolute stereochemistry of the phytochrome chromophore remains in doubt. These questions will be addressed in a future report on the stereochemistry of biliprotein chromophores.

Phototransformation Mechanism $P_R \rightleftharpoons P_{FR}$. Many different structural possibilities have been proposed to account for the P_R to P_{FR} phototransformation. Most of these hypotheses are incompatible with the experimental evidence from the present study. Nonetheless, the proposals illustrated in Scheme I[4a,b,e,19–21] have all received support from quantum mechanical calculations to explain the spectral differences of P_R to P_{FR}.[22–24] A number of postulates,[19,20,4a,b] Scheme Ia–d, respectively, are irreconcilable with the ^{1}H NMR spectral data of the phytochromobilipeptides

(17) Data obtained from phycobilipeptides in addition to β_1-phycocyanobiliheptapeptide **1**[5] (in preparation).

(18) The coupling constant of 5.0 Hz which we observe for $^3J_{2H-3H}$ in both **1** and **2** agrees well with the value of this coupling constant in trans succinimide models, the cis coupling constant being somewhat larger (unpublished work, this laboratory). See: (a) Klein, G.; Rudiger, W. *Justus Liebigs Ann. Chem.* **1978**, 267. (b) Schoch, S.; Klein, G.; Linsenmeier, U.; Rudiger, W. *Ibid.* **1976**, 549. (c) Ref 5 for discussion of the stereochemistry of **1**.

(19) Siegelman, H. W.; Chapman, D. J.; Cole, W. J. In "Porphyrins and Related Compounds"; Goodwin, T. W., Ed., Academic Press: New York, 1968, pp 107–20.

(20) Crespi, H. L.; Smith, U.; Katz, J. J. *Biochemistry* **1968**, *7*, 2232.

(21) (a) Song, P.-S; Chae, Q.; Gardner, J. D. *Biochim. Biophys. Acta* **1979**, *576*, 479. (b) Song, P.-S.; Chae, Q. *Photochem. Photobiol.* **1979**, *30*, 117.

(22) Burke, M. J.; Pratt, D. C.; Moscowitz, A. *Biochemistry* **1972**, *11*, 4025.

(23) Pasternak, R.; Wagniere, G. *J. Am. Chem. Soc.* **1979**, *101*, 1662.

(24) Sugimoto, T.; Oishi, M.; Suzuki, H. *J. Phys. Soc. Jpn.* **1977**, *43*, 619 and previous papers in the series.

Scheme I. Previous Hypotheses for P_R to P_{RF} Phototransformation[a]
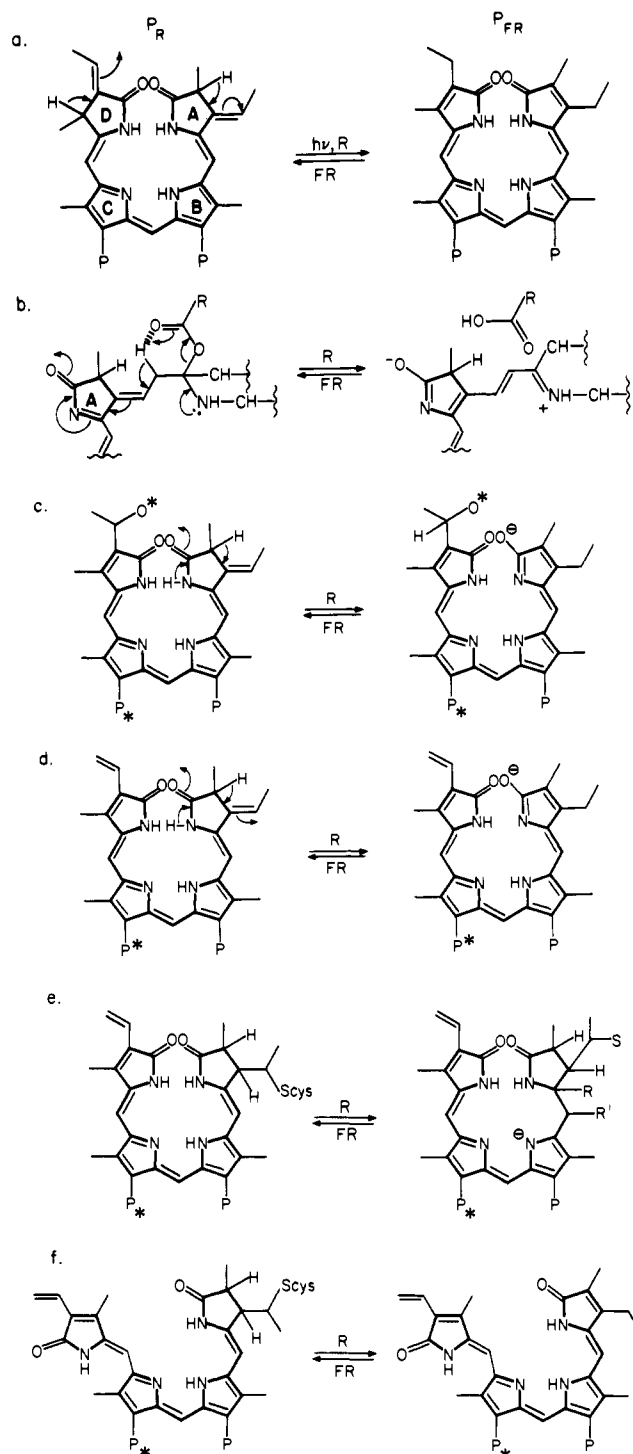
[a] (a) Reference 19. (b) Reference 20. (c) Reference 4a. (d) Reference 4b. (e) Reference 4e. (f) Reference 21. (Asterisks represent covalent linkages to the apoprotein. These structures are written in the cyclic form and are not meant to suggest proposed conformations except in f. P represents a propionic acid side chain.)

2 and **3**. On the other hand, P_R chromophore structures which are consistent with our experimental results have been proposed in the two models for phytochrome phototransformation,[4e,21] shown in Scheme Ie,f. These proposals differ with respect to the structure of P_{FR} where Klein et al.[4e] propose an anionic biliviolin chromophore, and Song et al.[21] suggest a biliverdin type prosthetic group for P_{FR}. Both of these models for P_{FR} are based on spectral analysis of native and denatured P_{FR} and have stimulated theo-

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rectical and spectroscopic analysis of model compounds, including various tripyrrinones such as mesobiliviolin 5,[4c,23] and biliverdin 6[21,22,25] in support of the former[4e] and latter hypotheses,[21] respectively.

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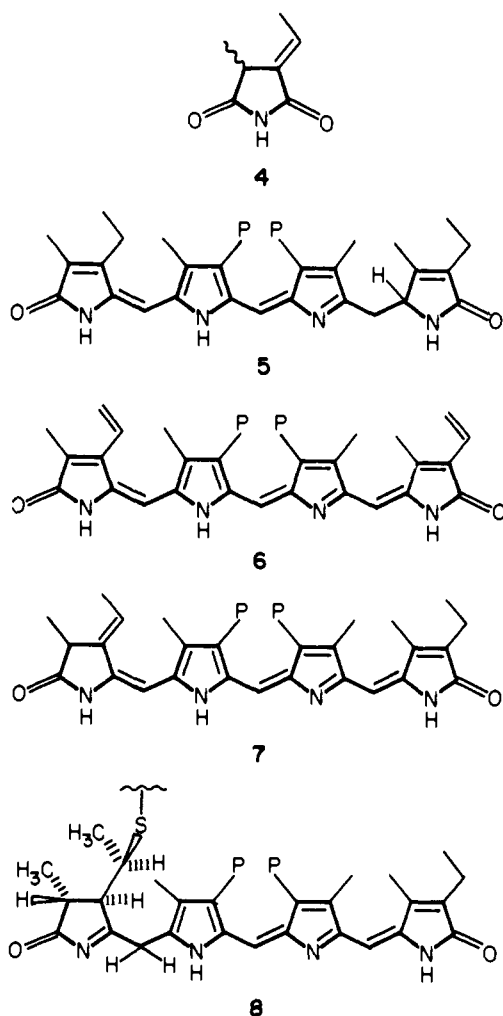
The proposal of an anionic biliviolin structure for the P_{FR} chromophore (Scheme Ie) has been based on the observation that denaturation of P_{FR} in acidic 8 M guanidinium chloride produced a biliviolin species (λ_{max} ~610 nm, pH 1.5).[4c] That oxidative photodimerization of dihydrobiliverdin model compounds affords biliviolin pigments has been suggested as a molecular model for $P_R \rightleftharpoons P_{FR}$ phototransformation.[26] The occurrence of two chromophores in the phytochrome apoprotein is incompatible with our results and those of others,[2] however. Furthermore, on the basis of pK_a considerations, it is difficult to conceive of a chromophore–protein interaction which would stabilize the anionic biliviolin structure for P_{FR} proposed in Scheme Ie. Theoretical calculations have cast considerable doubt on the validity of this proposal.[21]

In this study we have observed the incorporation of deuterium at the C-5 methine bridge of the phytochromobilin prosthetic group. The exchange of C-5 H with deuterium in β_1-phycocyanobiliheptapeptide (1) observed in an earlier study led to a mechanistic proposal for the elimination of the blue pigment 7 from *C*-phycocyanin during methanolysis.[5] The proposed intermediate 8 in this process, an isomer of 1, has a biliviolin-type chromophoric system, a fact we used to explain the occurrence

of purple pigments (λ_{max} ~590 nm, pH ≤2) which often accompany the purification procedure. Other explanations for the occurrence of these purple pigments have been proposed, since bilitriene model compounds show a pronounced tendency to photoisomerize[27,28] and also to react with nucleophiles in the presence of light[29] producing pigments with blue spectral shifts. The biliviolin-type spectrum obtained after denaturing P_{FR} in acidic 8 M guanidinium chloride[4c] could be due to an artifactual isomerization product derived from the native P_{FR} chromophore by any of these mechanisms. On the other hand, this spectral change was not observed in the studies of Fry and Mumford, who have isolated the spectrally identical bilitriene peptide from both P_R and P_{FR}.[6] Therefore these denaturation experiments[4e] need not be interpreted as indicating a difference in the P_R and P_{FR} chromophoric systems as shown in Scheme Ie, but instead could indicate that the P_{FR} chromophore is more susceptible to chemical (or photochemical) reactions during denaturation than the P_R chromophore.

While transformation from a dihydrobiliverdin to a biliverdin-type chromophore as proposed in Scheme If[21] seems reasonable, the reverse conversions appear to be energetically unfavorable. The reported instability of the P_{FR} form of phytochrome and its reversion to P_R is not consistent with this proposal, neglecting any protein stabilization. Furthermore, this proposal of Scheme If requires the cleavage of the thioether linkage during phototransformation. On the basis of our experimental evidence for a single linkage, that of the thioether, this mechanism would lead to the complete covalent release of the phytochrome chromophore from the apoprotein during phototransformation. Therefore, in principle, this P_{FR} chromophore should be easily extractable with organic solvents. There is no indication from other studies on phytochrome that this is the case.[2]

It is clear from these considerations that all of the proposed mechanisms shown in Scheme I are inadequate representations of the chromophore interconversions which occur during phytochrome phototransformation. We suggest three hypotheses (Scheme IIa–c)[30] for the phototransformation of phytochrome based on the above considerations and the evidence presented in this report. These mechanisms show the chromophoric system in linear conformations for simplicity. We do not intend to imply the absence of conformational changes of the chromophore accompanying phototransformation, which is an area of controversy among theoreticians.[21–24] The bond making–bond breaking hypothesis of Scheme IIa has support from studies which show that 1.7 additional cysteine-SH groups become accessible to sulfhydryl reagents after P_R to P_{FR} phototransformation.[31] Whether this indicates the cleavage of the cysteine thioether linkage remains to be determined. To give the spectral shift for P_{FR} and to ensure the P_{FR} is covalently linked, the Schiff base type linkage, perhaps via lysine or histidine residues, is proposed in Scheme IIa.

In Scheme IIb the spectral red shift for P_{FR} is rationalized by means of an acyl enol linkage (perhaps via aspartic acid or glutamic acid) giving rise to an additional double bond in the chromophoric system, as well as a positive charge migration from

(25) (a) Lehner, H.; Braslavsky, S. E.; Schaffner, K. *Justus Liebigs Ann. Chem.* **1978**, 1990. (b) Holzwarth, A. R.; Lehner, H.; Braslavsky, S. E.; Schaffner, K. *Ibid.* **1978**, 2002. (c) Margulies, L.; Stockburger, M. *J. Am. Chem. Soc.* **1979**, *101*, 743. (d) Sheldrick, W. S. *J. Chem. Soc.* **1976**, 1457.
(26) (a) Scheer, H. *Z. Naturforsch., C* **1976**, *31*, 413. (b) Scheer, H.; Krauss, C. *Photochem. Photobiol.* **1977**, *25*, 311.

(27) Falk, H.; Grubmayr, K.; Haslinger, E.; Schlederer, T.; Thirrung, K. *Monatsh. Chem.* **1978**, *109*, 1451.
(28) We also observed a nearly complete phototransformation of the β_1-phycocyanobiliheptapeptide (1) in the CD spectrometer from a blue form (λ_{max} 650 nm in anhydrous trifluoroacetic acid) to a purple form (λ_{max} 590 nm). The characterization of this purple pigment and its relevance to the proposed C-5 isomerization hypothesis is currently being studied.
(29) Krauss, C.; Bubenzer, C.; Scheer, H. *Photochem. Photobiol.* **1979**, *30*, 473.
(30) In Scheme II we have chosen to represent the P_R form of phytochrome as the protonated structure. Spectrophotometric titration has shown the pK_a of denatured P_R phytochrome to be 5.4.[4c] Furthermore the similarity of the absorption spectral properties of phytochromobiliundecapeptide (2) and denatured P_R[4c] in acidic solvent (λ_{max} 665 nm in 0.01 N trifluoroacetic acid, Figure 4) with those of native P_R phytochrome (λ_{max} 662 nm, Figure 1) suggests that the phytochrome chromophore is protonated. The difference in the absorption spectrum of P_R with that of the free base of 2 (λ_{max} 600–610 nm) is dramatic.
(31) Hunt, R. E.; Pratt, L. H. Abstracts of the 7th Annual American Society for Photobiology, 1979, June 24–28, Asilomar Conference Grounds, Pacific Grove, CA.

Scheme II. New Proposals for P_R to P_{FR} Phototransformation[a]

a.

b.

c.
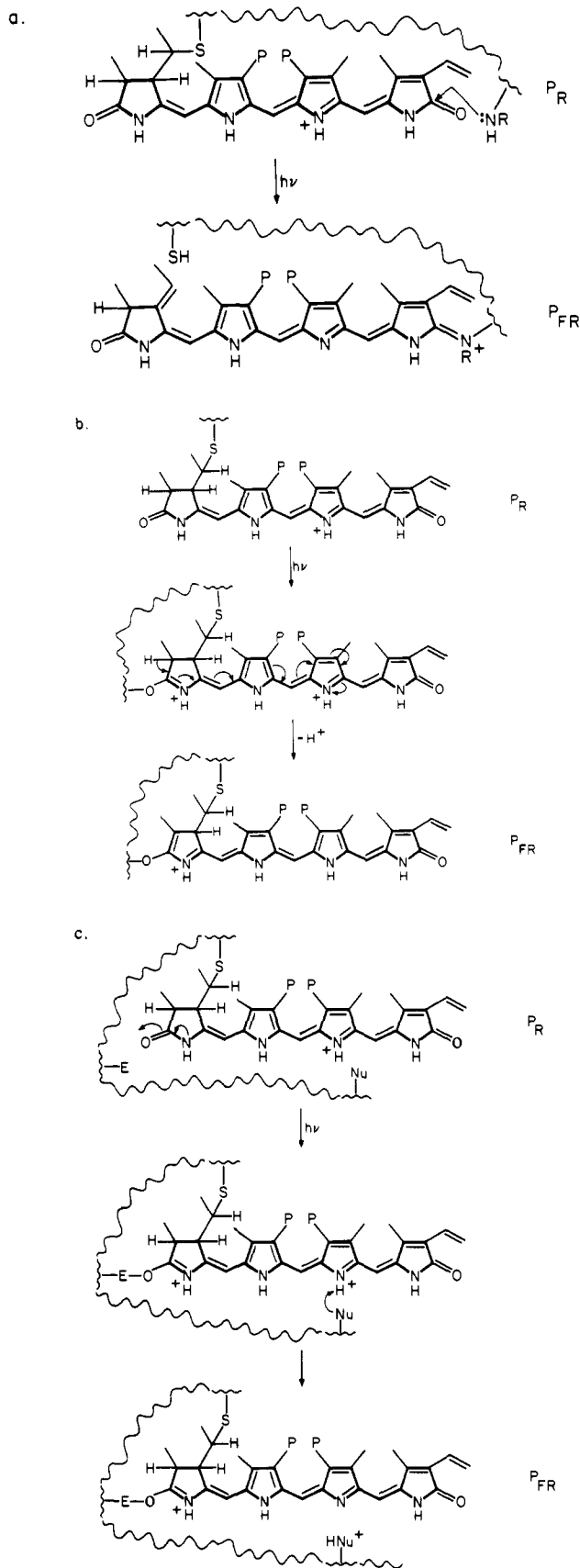
a (a) With thioether cleavage and Schiff base formation. (b) With enol acylation and migrating positive charge. (c) With lactam–lactim interconversion accompanied by deprotonation.

ring C to ring A. Both of these hypotheses suggest experiments which can establish the nature of any new chromophore–protein linkages, and such experiments are actively being considered.

A third postulate for phytochrome phototransformation is shown in Scheme IIc. This mechanism results in the movement of positive charge from the center of the chromophoric system to the terminal ring A as well as an increase in conjugation by lactam–lactim interconversion. Accompanying this reorientation of charge is the change in association of the chromophore with the apoprotein. One possibility for this association could involve the imidazole side chains of histidine, which can act as both a nucleophile (Nu) and an electrophile (E). With this mechanism, the migration of charge from ring C to ring A is possible via the intermediacy of a single histidine imidazole residue. Furthermore, the loss of a proton from ring C during phototransformation to P_{FR} would remove the steric effect restricting partial cyclization of the bilitriene,[32] a possible mechanism of photoconversion.

Because of the instability of the P_{FR} form of phytochrome, indicated by its reversion to P_R under a variety of conditions, and the fact that small chromopeptides derived from phytochrome are not photoreversible,[2,6] it is generally accepted that the role of the apoprotein in stabilizing the P_{FR} chromophore is an important one. Recent evidence from protein surface labeling experiments of highly purified phytochrome has shown differences in the surface properties of P_R and P_{FR}.[31] Selective chemical modification of the P_R and P_{FR} chromophores in a similar manner might provide some understanding of the differences in the chemical and physical association of the phytochrome chromophore with the apoprotein. Such experiments also are being considered.

Experimental Section

Materials. Oats (*Avena sativa* L., cv. Garry), obtained from Whitney, Dickinson Seeds, Inc. Buffalo, NY, were grown and harvested as previously described[7] and stored at −20 °C until extracted. Pepsin (Worthington Biochemicals, Anson activity grade 2500–3000 units) and thermolysin (Sigma Chemical Co., activity 63 units/mg of solid) were used for proteolysis experiments. High-performance liquid chromatography grade CH_3CN from Burdick and Jackson and water purified with a Milli-Q system (Millipore Corp.) were used for high-performance liquid chromatography.

Instrumentation. ^1H NMR spectra of the chromopeptides were taken in [$^2H_5$]pyridine solution at 23–25 °C on a homemade spectrometer.[33] High-performance liquid chromatography was done with a Spectra Physics 3500 B instrument equipped with a Schoeffel 770 variable-wavelength detector. Absorption spectra and phytochrome spectral assays were taken on a modified Cary 118 spectrometer.[8] Amino acid analyses were performed on a Beckman 120C analyzer by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley. Amino acid sequence analyses were obtained on a Beckman 890C sequencer by Dr. Al Smith at the Department of Biochemistry and Biophysics, University of California, Davis.

Phytochrome Preparation. The handling of plants and the purification procedure were performed under green safelight.[34] Undegraded phytochrome (M_r 120 000) was repetitively isolated from 4-kg batches of etiolated oat seedlings according to the method of Hunt and Pratt.[7] Crude extracts were partially purified by brushite chromatography (bed volume ~1.5 L, 13-cm diameter).[7] After spectral assay,[8] the phytochrome-containing fractions (>5 μg/mL) were combined and precipitated with 200 g/L solid $(NH_4)_2SO_4$. The precipitate was then resuspended in ~50 mL of 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.8, and measured by spectral assay.[8] The typical yield of brushite-prepared phytochrome from 4 kg of etiolated oats was 50–60 mg (SAR = 0.07, Figure 1). After a saturating far-red irradiation (Sylvania 150-W floodlamp impinging on an Optical Industries 720-nm interference filter

(32) In general the free bases of bilitrienes assume more cyclic conformations than those of the protonated forms as shown by the decrease in the ratio of the red absorption to the blue absorption bands of bilitrienes upon deprotonation. This is shown more elegantly by MO calculations in ref 22–25.

(33) The NMR spectrometer was designed and constructed by Dr. Willy C. Shih, Laboratory of Chemical Biodynamics, University of California, Berkeley. Instrumentation documentation is provided in: Shih, W. C. Ph.D. Thesis, 1979, University of California, Berkeley.

(34) The green safelight used for harvesting oats and for phytochrome isolation was obtained by wrapping green fluorescent tubes (Sylvania No. F40 Green) with one sheet each of a medium-blue-green plastic (Roscolene No. 877) and medium-green plastic (No. 874) available from Rosco Laboratories, Hollywood, CA.

with a 10-nm bandwidth), the brushite–phytochrome solution was stored frozen at −20 °C in the dark.

Phytochromobilipeptides. Pepsin Digestion. This procedure was performed under green safelight[34] at 4 °C by a modification of the published procedure.[6] Brushite P_R phytochrome (614 mg, 5.1 μmol, SAR = 0.06) in 1 L of 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.8, was precipitated with 300 g of solid $(NH_4)_2SO_4$. After centrifugation (15 min, 20000g) the pellet was suspended in 1.3 M HCOOH (200 mL) and stirred overnight. Nine hours later this suspension was centrifuged (15 min, 20000g). The precipitate was resuspended in 1.3 M HCOOH (131 mL) to which a solution of pepsin (32 mL, 8.9 mg/mL in 1.3 M HCOOH) was added. The mixture was then incubated with stirring for 4.5 h at 37 °C under Ar. After digestion, the mixture was centrifuged (15 min, 20000g), rotary evaporated to 25 mL, and applied to a BioGel P4 column (2.5 × 33.5 cm, flow rate 45 mL/h; preequilibrated with 1.3 M HCOOH).[35] The column was then washed with 550 mL of 1.3 M HCOOH while most of the blue material remained adsorbed to the first two-thirds of the column. This blue fraction was eluted from the column with 25% aqueous HOAc and collected in a 168-mL volume, and from its absorption spectrum represents 3.1 μmol (58% yield) of phytochromobilin (with $\epsilon_{665nm} = 3.2 \times 10^4$ L mol^{-1} cm^{-1}).[6] The amino acid composition of this fraction is compiled in Table I. The ^{1}H NMR spectrum is illustrated in Figure 1 of the supplementary material.

Thermolysin Digestion. To minimize photochemical side reactions, this procedure was performed under green safelight[34] or in the dark whenever possible. The pepsin-cleaved phytochromobilin peptide fraction was lyophilized in two equal portions in 5-mL ampules. The dry, blue residues were then dissolved in 1.0 mL of 0.1 N NH_4HCO_3 to which 200 μL of thermolysin solution (0.51 mg/mL in 0.1 N NH_4HCO_3) was added. After freeze–thaw degassing twice, the ampules were sealed

under vacuum. The mixtures were incubated at 37 °C for 4 h and cooled in ice; 300 μL of glacial acetic acid was introduced into each ampule.

The resulting dark blue solution was applied to a Sephadex G50 column (medium, 2.5 × 50 cm, flow rate 49 mL/h, preequilibrated with 25% aqueous HOAc) and eluted with 25% aqueous HOAc. A colorless 134-mL fraction was collected before the phytochromobilipeptide fraction eluted within 64 mL. On the basis of the absorption spectrum of this fraction, the recovery was determined as 2.4 μmol, 47% overall yield, of phytochromobilipeptides (see supplementary materials, Figure 2).

High-performance liquid chromatography of this thermolysin chromopeptide mixture was accomplished on a C_{18} reversed-phase column. As shown in Figure 3, five major phytochromobilipeptides, fractions 1–5, and three cleaved pigments, fractions 6–8, were obtained after high-performance liquid chromatography. In Tables I and II, the amino acid composition of fractions 2–5 and the sequence data for fraction 5 are tabulated. The absorption and ^{1}H NMR spectra of fraction 5, phytochromobiliundecapeptide (2), are illustrated in Figures 3–6 and Table III.

Supplementary Material Available: Full details of the ^{1}H NMR spectra of the peptide moiety of phytochromobiliundecapeptide (2), the pepsin peptide from phytochrome, and the phytochromobilioctapeptide (3) and the absorption spectrum of the chromopeptide fraction after thermolysin digestion (5 pages). Ordering information is given on any current masthead page.

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(35) The BioGel P4 column was prewashed with a mixture of 0.01 N EDTA and 0.1 N L-ascorbic acid to remove any trace metal or other oxidizing contaminants in the gel.

Kinetics of Thiamine Cleavage by Bisulfite Ion

Daniel R. Doerge and Lloyd L. Ingraham*

Contribution from the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. Received August 13, 1979

Abstract: The rate of cleavage of thiamine by bisulfite ion was found to be dependent on the square of the concentration of bisulfite ion at low concentrations of bisulfite. These results support the Zoltewicz–Kaufmann mechanism for thiamine cleavage in which the second step is an S_N2 displacement by bisulfite ion. Support is also given to the Zoltewicz–Kauffman suggestion that the enzyme thiaminase utilizes a similar mechanism in thiamine cleavage by amines.

Introduction

The bisulfite cleavage of thiamine has been known since 1935.[1] The products of the reaction are 2-methyl-4-aminopyrimidyl-5-methanesulfonate (V) and 4-methyl-5-(2-hydroxyethyl)thiazole. This reaction has been considered to be a simple displacement reaction by bisulfite ion for many years in spite of the fact that the reaction is unique for bisulfite ion and that the Japanese literature contained several reports that bisulfite ion catalyzed the cleavage by other nucleophiles, predominately amines.

Zoltewicz and Kauffman[2] have proposed a novel mechanism for the cleavage as a result of their studies of the bisulfite-catalyzed azide cleavage of thiamine. These authors proposed that the first step is a bisulfite addition to the 6-position of the pyrimidine ring (cf. Scheme I) followed by either an S_N1 or S_N2 displacement by azide ion or the formation of a sultone (III) followed again by reaction with azide ion. The Zoltewicz–Kauffman mechanism for bisulfite cleavage would replace the azide ion with bisulfite

ion and thus require two bisulfite ions for the reaction. Zoltewicz and Kauffman found a unimolecular dependence of the rate on the concentration of bisulfite ion as was found earlier by Leichter and Joslyn.[3] This result could be found for the sultone mechanism, the S_N1 mechanism, or an S_N2 mechanism in which the rate of reaction of II with bisulfite ion is faster than the dissociation of II to I and bisulfite ion.

In order to investigate the latter possibility, we have studied the kinetics at low concentrations of bisulfite ion where the reaction of II with bisulfite ion would be slower than the dissociation.

Experimental Section

Materials. All reagents were of analytical reagent purity.

Kinetic Procedure. Reactions were run in a sealed glass vessel maintained at 25.0 ± 0.2 °C by a Lauda circulating water bath. All reactions were carried out in 1 mM buffer (citrate for pH 3.5, 4.0, 4.5; acetate for pH 5.0; tris for pH 6.8) and pH values checked before and after each reaction. The pH values did not change within experimental error during the reaction. Sodium bisulfite (Mallinkrodt) solutions were prepared

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(1) Williams, R. R. *J. Am. Chem. Soc.* **1935**, *57*, 229.

(2) Zoltewicz, J. A.; Kauffman, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 3134.

(3) Leichter, J.; Joslyn, M. A. *Biochem. J.* **1969**, *113*, 611.