Phosphopeptide Mapping of *Avena* Phytochrome Phosphorylated by Protein Kinases in Vitro†

Robert W. McMichael, Jr., and J. Clark Lagarias*
Department of Biochemistry and Biophysics, University of California, Davis, Davis, California 95616
Received October 27, 1989; Revised Manuscript Received December 27, 1989

ABSTRACT: We previously demonstrated that protein kinases are useful probes of conformational changes that occur upon photoconversion of phytochrome [Wong, Y.-S., Cheng, H.-C., Walsh, D. A., & Lagarias, J. C. (1986) *J. Biol. Chem. 261*, 12089-12097]. Here we present phosphopeptide analyses of oat phytochrome phosphorylated by three mammalian protein kinases and by a polycation-stimulated, phytochrome-associated protein kinase. Phosphorylation of the Pr form by the cAMP-dependent protein kinase occurs predominantly on Ser_{17} while Ser_{598} is the preferred phosphorylation site on Pfr. The cGMP-dependent and Ca^{2+}-activated, phospholipid-dependent protein kinases, which phosphorylate only the Pr form of phytochrome, recognize the same region on the phytochrome polypeptide as the cAMP-dependent protein kinase. Polycation-stimulated phytochrome phosphorylation reveals that, in contrast to the mammalian enzymes, the plant kinase recognizes the serine-rich, blocked N-terminus of phytochrome. The potential regulatory role of phytochrome phosphorylation, particularly in the structurally conserved serine/threonine-rich N-terminal region of the phytochrome polypeptide, is suggested by these results.

The characteristic photoreversible red/far-red light regulation of plant growth and development by phytochrome is the most well-known of the photomorphogenetic receptor systems of plants (Shropshire & Mohr, 1983; Furuya, 1987). Despite the knowledge that photointerconversion between the red-absorbing Pr[1] form and the far-red-absorbing Pfr form initiates many photomorphogenetic responses in plant tissue, phytochrome-dependent signal transduction pathways are still poorly understood. One approach to molecular dissection of the signal transduction pathways has entailed identification and characterization of light-induced changes in the phytochrome molecule. The rationale for such experiments is that some of these regions of the photoreceptor are likely to participate in the interaction with regulatory molecules and/or with components of the signal transduction pathway. Experiments with chemical modification reagents, monoclonal antibodies, and protein-modifying enzymes have provided direct evidence for light-induced changes in phytochrome conformation [see review by Lagarias (1985), Song (1988), and Cordonnier (1989)]. Knowledge of specific regions of phytochrome structure that are important to function, however, is limited.

Protein phosphorylation is a particularly useful tool for protein structure–function studies because (1) modification conditions are usually mild enough to preserve the native protein structure, (2) modification is quite specific owing to the structural specificity inherent to an enzyme catalyst, and (3) modification is potentially reversible by using protein phosphatases. These attributes of protein phosphorylation have provided the impetus for further characterization of phytochrome phosphorylation reported in an earlier study (Wong et al., 1986). In the present study, we present a detailed phosphopeptide analysis of oat phytochrome phosphorylated by three mammalian protein kinases and a polycation-stimulated, phytochrome-associated protein kinase. These studies have defined the phosphopeptide substrate recognition sites for all four enzymes, which confirm the conclusion of our previous report that phytochrome photoconversion effects a significant change in photoreceptor conformation (Wong et al., 1986).

Materials and Methods

Materials. Trypsin (type XI, DPCC treated) from bovine pancreas, PITC, cGMP, 1,2-diolein, phosphatidylserine, and pentylagarose (catalog no. P9658) were obtained from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) as the triethylammonium salt in aqueous solution with 5 mM 2-mercaptoethanol was purchased from Amersham. *N*-Methylmorpholine (bp 116 °C) from Mallinckrodt and sequanal-grade TFA (bp 71 °C) from Pierce were redistilled before use. DABITC was obtained from Pierce. Acetonitrile, 85% (w/v) H_3PO_4, and KH_2PO_4 (all HPLC grade) were obtained from Fisher. The 100 mM potassium phosphate, pH 2.1, buffer was prepared by mixing 100 mmol each of H_3PO_4 and KH_2PO_4 in a final volume of 2 L of Milli-Q H_2O (Millipore), and the 100 mM potassium phosphate (pH 6.8) buffer was prepared by titrating 100 mmol of KH_2PO_4 with solid potassium hydroxide to pH 6.8. Both solutions were filtered through a NORGANIC cartridge (Millipore) before use. All glassware was treated with 20% (v/v) nitric acid and rinsed extensively with Milli-Q H_2O. A 100% TCA stock solution was prepared by adding 227 mL of Milli-Q H_2O to the contents of an unopened 500-g bottle of TCA. The stock was aliquoted and stored at −20 °C.

Phytochrome Preparations. Phytochrome preparations with SAR values between 0.98 and 1.03 were obtained according to the method of Lagarias and Mercurio (1985) with the following two modifications. Prior to being loaded onto the

† This work was supported in part by the National Science Foundation (DCB87-04266). R.W.M. is a recipient of a Jastro–Shields graduate research award (University of California, Davis).

[1] Abbreviations: DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; HPLC, high-performance liquid chromatography; Pr, red light absorbing form of phytochrome; Pfr, far-red light absorbing form of phytochrome; PITC, phenyl isothiocyanate; SAR, specific absorbance ratio A_{668}/A_{280} for Pr; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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pentylagarose column, the clarified, resuspended ammonium sulfate pellet was diluted with 0.8 volume, instead of 0.9 volume, of 50 mM Tris-HCl buffer, pH 7.8, containing 2 M ammonium sulfate, 25% (v/v) ethylene glycol, and 1 mM EDTA. In addition, the particular pentylagarose resin used in these studies (Sigma P9658) differs from that previously used (Sigma P5393) with regard to the mode of linkage between the C5-ligand and the agarose matrix.

Protein Kinase Preparations. Purified cAMP-dependent protein kinase, catalytic subunit (kinase A), a gift from Dr. D. A. Walsh (Department of Biological Chemistry, University of California, Davis, CA), was isolated from bovine cardiac muscle (Fletcher et al., 1986). Purified bovine lung cGMP-dependent protein kinase (kinase G) was a gift of Dr. Donald Glass (Department of Pharmacology, Emory University School of Medicine, Atlanta, GA) (Glass & Krebs, 1979). The Ca^{2+}-activated phospholipid-dependent protein kinase (kinase C), a generous gift of Drs. Ronald H. Cooper and Judith L. Turgeon (Department of Human Physiology, University of California, Davis), was purified from rabbit brain by a modified method of Kikkawa et al. (1983).

In Vitro Phosphorylation and Trypsin Digestion of Phytochrome. In vitro phosphorylations of phytochrome were performed at 30 °C using conditions similar to those described earlier (Wong et al., 1986). The reaction buffer contained 50 mM Hepps/Tris, pH 7.8, 5 mM $MgCl_2$, 1.4 mM 2-mercaptoethanol, 0.2 mM EDTA, and 0.1 mM [γ-^{32}P]ATP with a specific activity of approximately 500 cpm/pmol. For the kinase G and kinase C experiments, the appropriate cofactors were added to the assay buffer as described previously (Wong et al., 1986). Phytochrome concentrations ranged from 0.8 to 3.2 μM (0.1–0.4 mg/mL) as calculated from the absorbance at 668 nm and the extinction coefficient of 1.32×10^5 M^{-1} cm^{-1} (Lagarias et al., 1987a). Kinase A, G, and C concentrations in the assay mixtures were 20, 10, and 1.3 μg/mL, respectively. Phosphorylation reactions were initiated by the addition of [γ-^{32}P]ATP. Following incubation, aliquots of the reaction mixtures were taken for determination of the percent molar incorporation using both a filter binding assay (Corbin & Reimann, 1975) and SDS–PAGE (see below). For peptide mapping experiments, the reaction was terminated by addition of cold 100% (w/v) TCA to a final concentration of 10%, and the protein precipitate was collected by centrifugation. Prior to trypsin digestion, TCA was extracted from the precipitate by washing as described by Garrison (1983), and the washed precipitate was resuspended in 50 mM *N*-methylmorpholine acetate, pH 7.8, buffer (0.75 mL/mg of protein). Trypsin was added to give 1:50 (w/w) ratio of trypsin to phytochrome. The sample was incubated at 37 °C for 18 h with continuous end-over-end mixing. After digestion was complete, the samples were evaporated to dryness with a Savant Speed Vac vacuum centrifuge.

Phosphorylation and Trypsin Digestion of Synthetic Peptides. In vitro phosphorylations of synthetic peptides, SP2 (R_{12}NRQSSQARVLAQTTLD$_{28}$) and SP6 (K_{592}PKREASLDEQIGDLK$_{607}$), were performed similarly to that described for phosphorylation of phytochrome. Peptide concentrations were 5 mg/mL, and all incubations were performed for 1 h at 30 °C. Phosphopeptide mixtures were then digested by the addition of trypsin to a 1:20 (w/w) ratio of trypsin to peptide followed by incubation at 37 °C for 18 h with continuous end-over-end mixing. Digestions were terminated by addition of acetic acid to 30% by volume, and the phosphopeptides were then separated from ATP and phosphate salts by passage through a 0.7 × 3.0 cm column of AG1-X8 resin (Bio-Rad) equilibrated in 30% acetic acid (Kemp et al., 1975).

Phosphopeptide Purification. Dried phosphopeptide digests were resolubilized in 100 mM HPLC-grade potassium phosphate, pH 2.1, buffer (100 μL/mg of phytochrome) followed by centrifugation for 10 min at 15600*g* to remove insoluble material. The supernatant was applied to a C18 reversed-phase column (0.46 × 15 cm, Beckman Ultrasphere ODS with a 0.46 × 2.5 cm guard column) which was equilibrated in 100 mM potassium phosphate buffer, pH 2.1. A 120-min linear gradient from 0% to 60% acetonitrile with a flow rate of 1 mL/min was used to elute peptides derived from phytochrome phosphorylated as Pr. Pfr-derived peptides were separated by using an identical gradient except that the rate of change in the acetonitrile concentration from 0% to 10% was increased to 1.0% per minute, and from 10% to 20% acetonitrile, the rate of change was lowered to 0.33% acetonitrile per minute. The column eluate was monitored for the absorbance at 230 nm, and 1.0-mL fractions were collected.

Radioactivity in the HPLC fractions was determined by Cerenkov counting (Mardh, 1975) with a Beckman LS 3801 liquid scintillation counter. Radioactive fractions were pooled and dried in a Savant Speed Vac vacuum centrifuge. Pooled fractions were dissolved in H_2O and applied to the same column equilibrated with 100 mM potassium phosphate buffer, pH 6.8. Pr-derived peptides were resolved by using a linear gradient from 0% to 10% acetonitrile over a 60-min period with a flow rate of 1 mL/min. Pfr-derived peptides were separated with a gradient of 5–18% acetonitrile over a 60-min period with a flow rate of 1 mL/min. Pooled phosphopeptide fractions isolated from this second HPLC system were dried under vacuum and dissolved in 0.1% TFA before being applied to a third HPLC column (0.46 × 15 cm, Ultramex C18, Phenomenex) equilibrated in 0.1% TFA. Pr-derived peptides were resolved by using a linear gradient from 0% to 10% acetonitrile over a 60-min period with a flow rate of 1 mL/min. Pfr-derived peptides were separated with a gradient from 14.5% to 21% acetonitrile over a 60-min period with a flow rate of 1 mL/min.

Acid Hydrolysis and Amino Acid Composition Analysis of HPLC-Purified Phosphopeptides. HPLC-purified phosphopeptide fractions in 2-mL glass ampules were dried in a Speed Vac vacuum centrifuge. Constant-boiling HCl (0.5 mL, Pierce, Sequanal Grade) containing 25 μL of 5% (w/v) phenol was added to the dry peptide residue. The samples were degassed twice by freeze-thawing, sealing under vacuum, and hydrolyzed at 110 °C for 24 h. Following hydrolysis, the samples were evaporated to dryness in a Speed Vac vacuum centrifuge. Amino acid composition analyses were performed on a Beckman 6300 and Varian precolumn FMOC derivatization, postcolumn fluorescence detection amino acid analyzers at the U. C. Davis Protein Structure Laboratory.

Edman Degradation Analysis. HPLC-purified phosphopeptides were dried under vacuum and subjected to repeated cycles of manual Edman degradation using the DABITC/PITC double coupling method (Chang et al., 1978). At the end of each cycle, an aliquot of the aqueous phase was removed and assayed for the presence of free phosphate, which was released from the amino-terminal phosphoserine via β-elimination during the cyclization of the PTH derivative (Wittmann-Liebold et al., 1986), by thin-layer electrophoresis at pH 3.5 on Eastman Kodak cellulose thin-layer plates (Cooper et al., 1983).

SDS–Polyacrylamide Gel Electrophoresis and Autoradiography. Discontinuous SDS–PAGE on 0.8-mm slab gels

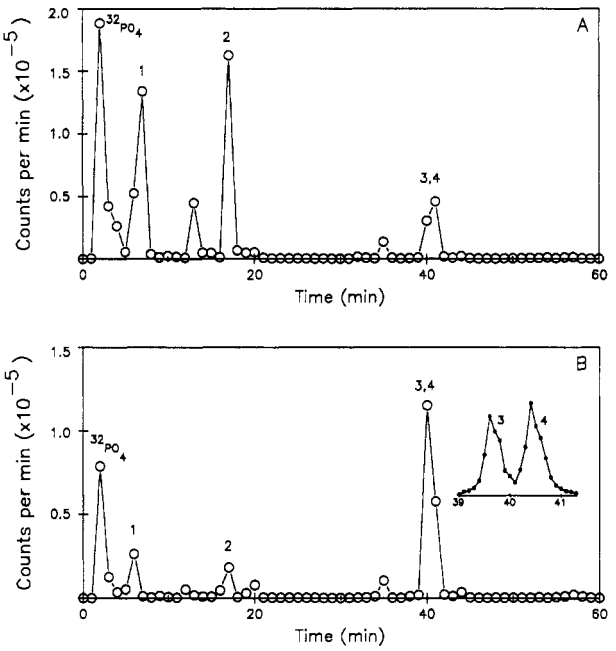

FIGURE 1: Phosphopeptide profiles of trypsin-digested phytochrome phosphorylated by the cAMP-dependent protein kinase. Complete trypsin digestion mixtures of phosphorylated phytochrome were separated on a reversed-phase C18 HPLC column using a 120-min linear gradient of acetonitrile and 0.1 M potassium phosphate, pH 2.1, as described under Materials and Methods. Labeled phosphopeptide profiles of phytochrome phosphorylated as Pr or Pfr by the cAMP-dependent protein kinase are shown in panels A (Pr) and B (Pfr). The inset in panel B shows the region from 39 to 41 min expanded to provide resolution of peaks 3 and 4.

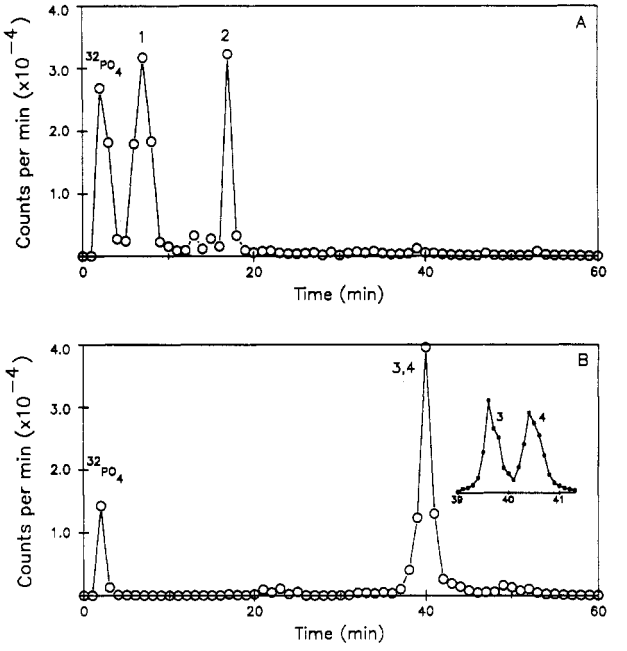

FIGURE 2: Phosphopeptide profiles of trypsin-digested synthetic peptides phosphorylated by the cAMP-dependent protein kinase. Digest mixtures of the synthetic peptides SP2 (R_{12}NRQSSQARVLAQTTLD$_{28}$) and SP6 (K_{592}PKREASLDEQIGDLK$_{607}$) were separated by reversed-phase HPLC under the same conditions described in Figure 1. Labeled phosphopeptide profiles for the digests of phosphorylated SP2 and SP6 are shown in panels A and B, respectively.

utilized 3% stacking, 7.5% resolving gels, and the Laemmli buffer system (Laemmli, 1970). Gels were prepared for autoradiography and quantitation by scintillation spectrometry as described previously (Wong et al., 1986).

Results

Avena phytochrome is a large protein, consisting of over 1100 amino acid residues (Vierstra & Quail, 1983). Identification of the in vitro phosphorylation sites on phytochrome recognized by various protein kinases therefore required the development of methodologies to resolve the complex mixture of polypeptides obtained upon terminal trypsin digestion. Purified phosphopeptides were obtained by three successive reversed-phase HPLC separations: a low-pH phosphate buffer system followed by a neutral pH phosphate buffer system and then a 0.1% TFA system. The structure of the pure phosphopeptides could be inferred from their amino acid compositions by comparison with the known primary structure(s) of *Avena* phytochrome (Hershey et al., 1985).

By use of this protocol, comparative phosphopeptide mapping of *Avena* phytochrome phosphorylated as Pr and Pfr was undertaken. The mammalian cAMP-dependent protein kinase (catalytic subunit) was used in these studies because it phosphorylates both forms of phytochrome to high levels (Wong et al., 1986). Initial analyses of digest mixtures of kinase A phosphorylated phytochrome, using the pH 2.1 phosphate buffer system, showed that the distribution of radiolabel in the eluted peptides differed for the samples phosphorylated as Pr or Pfr (Figure 1). While both samples contained four major phosphopeptides (labeled 1–4) and two minor phosphopeptides, most of the radiolabel associated with the Pr sample was found in peptides 1 and 2 (Figure 1A), while peptides 3 and 4 (Figure 1B and inset) contained most of the radiolabel associated with the Pfr sample. The possibility that these differences reflect incomplete trypsin digestion of the samples was investigated by extending the digestion periods and/or by adding fresh trypsin to the mixtures. Elution profiles remained unchanged in such experiments, indicating that the data shown in Figure 1 reflect true differences in the location of the phosphorylations on Pr and Pfr (data not shown).

Comparison of the known primary structure of *Avena* phytochrome (Hershey et al., 1985) and the known specificity of the cAMP-dependent protein kinase (Williams, 1976) revealed possible candidates for the phosphorylation sites on phytochrome utilized by this kinase. With the use of this information, a number of peptides were synthesized (Lagarias et al., 1987b), and two of these, SP2 (R_{12}NRQSSQARVLAQTTLD$_{28}$) and SP6 (K_{592}PKREASLDNQIGDLK$_{607}$), proved to be substrates for kinase A in vitro. When these two phosphopeptides were exhaustively digested with trypsin and analyzed on the pH 2.1 phosphate buffer reversed-phase HPLC system, the radioactivity elution profiles for SP2 and SP6 (Figure 2) were in very good agreement with those observed for Pr and Pfr, respectively (Figure 1). These experiments showed that trypsin-digested, phosphorylated SP2 yielded two labeled peptides with elution positions identical with those of phosphopeptides 1 and 2 observed for the Pr digests (Figure 1A). The analysis of SP6 confirmed the presence of two phosphopeptides with elution positions identical with phosphopeptides 3 and 4 observed for the Pfr sample (Figure 1B).

The experiments with the peptide substrates strongly indicated that the serines of SP2 and SP6 represented the respective phosphorylation sites on Pr and Pfr. Which of the two serines on SP2 (and Pr) were phosphorylated and why two phosphopeptides were obtained from each of the two peptides (and from phytochrome) remained important unanswered questions. To resolve these issues, all four major phosphopeptides obtained by in vitro phosphorylation of *Avena* phy-

Table I: Yields of HPLC-Purified Phosphopeptides from Phytochrome Phosphorylated by Kinase A[a]

HPLC system[b]	Pr peak	Pr nmol[c]	Pr yield (%)	Pfr peak	Pfr nmol[c]	Pfr yield (%)
(1) 0.1 M KPO_4, pH 2.1	1	1.6	100	3	1.2	100
	2	0.9	100	4	1.6	100
(2) 0.1 M KPO_4, pH 6.8	1	0.75	47	3	0.67	56
	2	0.45	50	4	0.78	49
(3) 0.1% TFA	1	0.45	28	3	0.26	21
	2	0.24	27	4	0.38	24

[a] Analyses were performed using 8 nmol of Pr and 10 nmol of Pfr. [b] The gradient elution conditions for each separation step are described under Materials and Methods. [c] Yields in nanomoles were estimates from the total cpm in the peak and the specific activity of the [γ-^{32}P]ATP.

Table II: Amino Acid Composition Analysis for Phosphopeptides Derived from Phytochrome Phosphorylated as Pr by Kinase A

amino acid	peak 1 pmol	peak 1 exptl[a]	peak 1 predicted[b]	peak 2 pmol	peak 2 exptl[a]	peak 2 predicted[b]
Ser	475	1.9	2	251	2.1	2
Glx	545	2.2	2	285	2.4	2
Ala	218	0.9	1	166	1.4	1
Leu	20	0.1	0	20	0.2	0
Lys	25	0.1	0	27	0.2	0
Arg	246	(1)	1	117	(1)	1

[a] Values have been normalized to an arginine content of 1 residue per peptide. [b] Predicted number of residues in the hexapeptide $Q_{15}SSQAR_{20}$ based on the deduced primary structure of type 3 (and type 5) *Avena* phytochrome (Hershey et al., 1985).

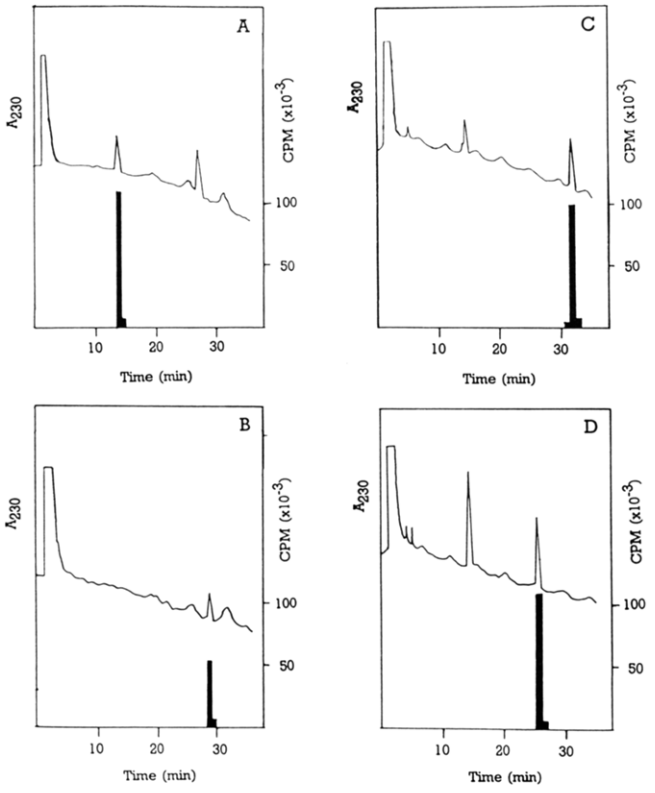

FIGURE 3: Final phosphopeptide profiles for the phosphopeptides derived from phytochrome phosphorylated by kinase A. Phosphopeptides were separated on the third (0.1% TFA) HPLC system as described under Materials and Methods. In each panel, the trace represents the absorbance at 230 nm, and the bars under each absorbance trace indicate the amount of radioactivity recovered in 1-mL fractions. Panels A–D illustrate the profiles for phosphopeptides 1–4, respectively, which are numbered as in Figure 1.

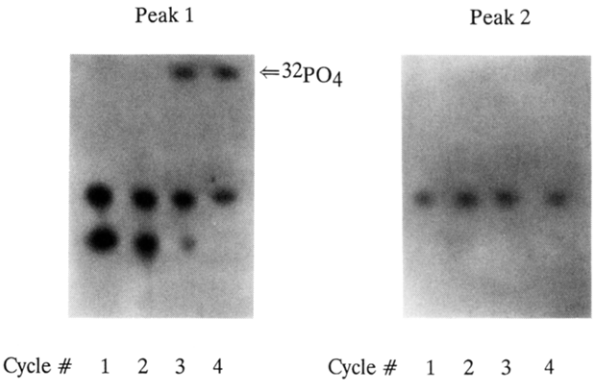

FIGURE 4: Edman degradation of phosphopeptides 1 and 2. Thin-layer electrophoresis was used to detect the release of free phosphate from Pr-derived phosphopeptides 1 and 2 (see Figure 1) as described under Materials and Methods. Shown here are the autoradiographs of the thin-layer plates for the analysis of phosphopeptide 1 (panel A) and phosphopeptide 2 (panel B). The cycle number is indicated below each sample.

tochrome (1–4, Figure 1) were purified and characterized. As outlined above, phosphopeptides 1 and 2, obtained from Pr-phosphorylated samples (Figure 1A), and phosphopeptides 3 and 4, obtained from Pfr-phosphorylated samples (Figure 1B), were each successively fractionated on the neutral pH phosphate buffer and 0.1% TFA buffer HPLC systems. The yields of each phosphopeptide on each column are shown in Table I. The elution profiles showing both A_{230} and radioactivity for the final HPLC column for each of the four peptides are illustrated in Figure 3 (A–D).

Amino acid analyses were performed on acid hydrolysates of each of the purified phosphopeptides (Tables II and III). These results show that the Pr-derived phosphopeptides (i.e., 1 and 2) have identical amino acid compositions. Both correspond well with the hexapeptide sequence, $Q_{15}SSQAR_{20}$, found in type 3 and type 5 *Avena* phytochromes (Hershey et al., 1985). The compositions of the two Pfr-derived phosphopeptides (i.e., 3 and 4) are quite similar, differing only in the occurrence of an arginine residue in phosphopeptide 3 (Table III). The composition of phosphopeptide 3 corresponds well with the sequence $R_{595}EASLDNQIGDLK_{607}$, based on the deduced primary structure of *Avena* phytochrome (Hershey et al., 1985). Except for the lack of the N-terminal arginine residue, phosphopeptide 4 corresponds to the same sequence on phytochrome. Since both peptides contain only a single serine residue, these data indicate that Ser_{598} is the predominant cAMP-dependent protein kinase phosphorylation site on Pfr.

The presence of two serine residues (i.e., Ser_{16} and Ser_{17}) in the Pr-derived phosphopeptides required additional exper-

Table III: Amino Acid Composition Analysis for Phosphopeptides Derived from Phytochrome Phosphorylated as Pfr by Kinase A

	peak 3 (residues/peptide)			peak 4 (residues/peptide)	
amino acid	pmol	exptl[a]	predicted[b]	pmol	exptl[a]	predicted[b]
Asx	334	2.8	3	393	2.9	3
Ser	115	1.0	1	145	1.1	1
Glx	225	1.9	2	275	2.0	2
Gly	154	1.3	1	187	1.4	1
Ala	121	1.0	1	143	1.1	1
Ile	101	0.8	1	126	0.9	1
Leu	222	1.8	2	261	1.9	2
Lys	121	(1)	1	135	(1)	1
Arg	116	1.0	1			0

[a] Values have been normalized to a lysine content of one residue per peptide. [b] Predicted number of residues in the peptides R_{595}EASLDNQIGDLK$_{607}$ and E_{596}ASLDNQIGDLK$_{607}$ based on the deduced primary structure of type 3 (and type 4) *Avena* phytochrome (Hershey et al., 1985).

iments to resolve which serine residue(s) was (were) phosphorylated. This was accomplished by using a manual Edman degradation procedure. The results of the thin-layer electrophoresis analyses of phosphopeptides 1 and 2 are shown in Figure 4. For phosphopeptide 1, liberation of $^{32}PO_4$ occurred during the third cycle of the Edman degradation. By comparison, no radiolabel was released during the analysis of phosphopeptide 2. These results indicate that Ser_{17}, but not Ser_{16}, is the phosphorylated residue on phosphopeptide 1 and that phosphopeptide 2 has a blocked N-terminus. In view of the identical amino acid compositions of phosphopeptides 1 and 2, these results are consistent with the cyclization of the N-terminal glutamine residue to a pyroglutamate residue to form peptide 2 during the digestion and/or isolation procedures. In support of this hypothesis, phosphopeptide 1 can be partially converted to phosphopeptide 2 under the conditions of high pH and high temperature utilized in the Edman degradation procedure (see Figure 4, the lower phosphopeptide is converted to the upper phosphopeptide). This conversion of phosphopeptide 1 to phosphopeptide 2 has also been confirmed by HPLC analysis (data not shown).

Phosphorylations of phytochrome by the cGMP-dependent and Ca^{2+}-activated, phospholipid-dependent protein kinases were next examined. In an earlier study, we showed that (1) these kinases preferentially phosphorylate Pr, (2) the phosphorylation sites were located in the N-terminal 10-kDa domain, and (3) both kinases phosphorylated Pr to a lesser extent than did kinase A (Wong et al., 1986). Figure 5 shows that the phosphopeptide profiles for phytochrome phosphorylated as Pr with kinase C (Figure 5B) and kinase G (Figure 5C) are indistinguishable from that for kinase A (Figure 5A). The major phosphopeptides (labeled 1 and 2 in Figure 5) for all three protein kinases also comigrated on the other HPLC systems described in this study (data not shown).

Phytochrome phosphorylation by the polycation-stimulated protein kinase activity which is associated with our purified phytochrome preparations was also analyzed. This protein kinase activity modifies phytochrome in the N-terminal 10-kDa domain. In contrast to the results with the mammalian kinases, however, Pr is only slightly preferred to Pfr as a substrate for this activity (Wong et al., 1986). As shown in Figure 6, the phosphopeptide profile of a Pr phytochrome preparation phosphorylated in the presence of polylysine (Figure 6A) is quite different from that observed with kinase A (Figure 6B). A phosphopeptide profile identical with Figure 6A was obtained regardless of whether Pr or Pfr was used as substrate (data not shown). Control experiments were also performed which showed that the observed differences in the phosphopeptide profiles were not due to incomplete trypsin digestion or to changes in the phosphopeptide elution positions caused by the presence of polylysine (data not shown).

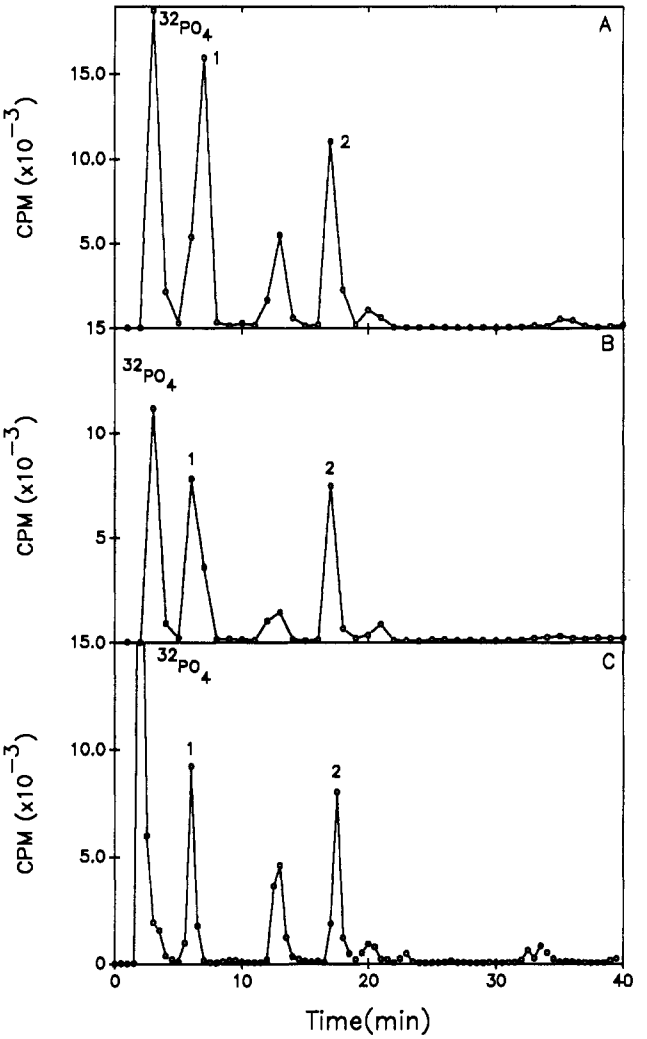

FIGURE 5: Phosphopeptide analyses of phytochrome phosphorylated by three mammalian protein kinases. Phosphopeptide profiles of eluted radioactivity for phytochrome samples phosphorylated by kinase A (100 μg of Pr; panel A), kinase C (500 μg of Pr; panel B), and kinase G (500 μg of Pr; panel C) using the 0.1 M KPO_4, pH 2.1, HPLC system as described under Materials and Methods are shown here.

The major phosphopeptide obtained from phytochrome phosphorylated by the polycation-stimulated protein kinase was further purified by using the other two HPLC systems described in this study. The amino acid composition of the purified peptide shown in Table IV indicates that the peptide is particularly serine-rich. Preliminary attempts at sequencing the peptide failed, indicating either that an insufficient amount of peptide was used or that the N-terminus of the peptide is blocked. These results are consistent with its assignment to the blocked N-terminal dodecapeptide,

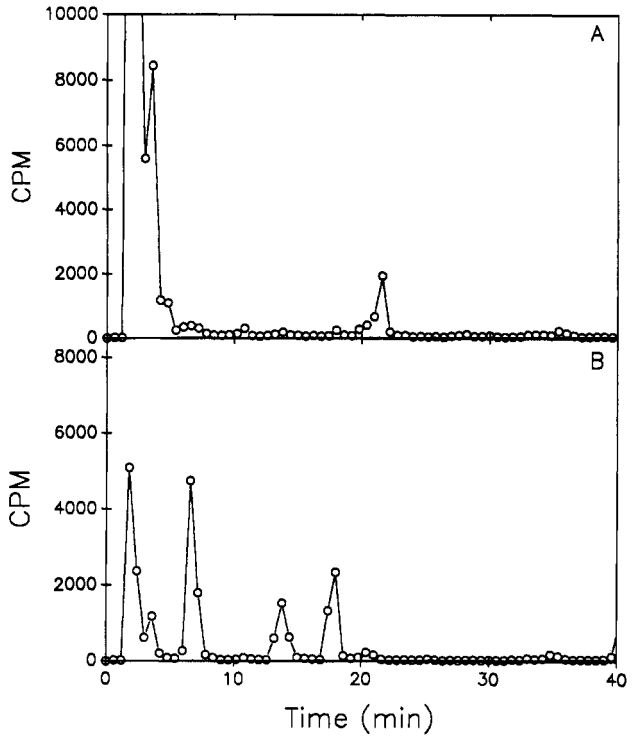

FIGURE 6: Comparative phosphopeptide mapping of phytochrome phosphorylated by kinase A and by the polycation-stimulated protein kinase. Phosphopeptide profiles of eluted radioactivity for phytochrome phosphorylated in the presence of polylysine (200 μg of Pr; panel A) or by kinase A (40 μg of Pr; panel B) using the 0.1 M KPO_4, pH 2.1, system as described under Materials and Methods are shown.

Table IV: Amino Acid Composition Analysis of the Major Phosphopeptide Derived from Phytochrome Phosphorylated by the Polycation-Stimulated Protein Kinase Activity[a]

amino acid	pmol	residues per peptide	
		exptl[b]	predicted[c]
Ser	416	7.6	8
Ala	65	1.2	1
Pro	ND[d]		1
Arg	110	(2)	2

[a] Composition was determined with a Varian precolumn FMOC derivitization, postcolumn fluorescence detection amino acid analyzer. Amino acids present at levels of less than 20 pmol have been omitted. [b] Values normalized to an arginine content of two residues per peptide. [c] Predicted number of residues in the dodecapeptide $NAcS_1SSRPASSSSSR_{12}$ based on the deduced primary structure of types 3, 4, and 5 *Avena* phytochromes (Hershey et al., 1985) and the mass spectral identification of *N*-acetylserine as the N-terminal residue (Grimm et al., 1988). [d] Not determinable due to the presence of an unidentified compound(s) which eluted near this amino acid. This also occurred for His and Gly which are not reported here.

$NAcS_1SSRPASSSSSR_{12}$, since the N-terminus of phytochrome is known to be *N*-acetylserine (Grimm et al., 1988).

Discussion

Phosphopeptide mapping studies on *Avena* phytochrome phosphorylated by protein kinases in vitro are in good agreement with previous investigations which show that a region near the N-terminus of the photoreceptor is exposed as Pr and that photoconversion to Pfr shields this region while exposing the central hinge region of the protein (Lagarias, 1985; Song, 1988; Cordonnier, 1989). When Pr is used as substrate for four different protein kinases examined here, phytochrome is phosphorylated at/or near the N-terminus. For the three mammalian enzymes, phosphorylation of Pr occurs on Ser_{17} (or possibly Ser_{16} for kinases C and G). The polycation-stimulated, phytochrome-associated protein kinase recognizes the serine-rich region at the immediate N-terminus of phytochrome. By contrast, while Pfr is a rather poor substrate for three of the four protein kinases studied here, kinase A phosphorylates Pfr on Ser_{598} which is far removed from the N-terminus. This evidence for light-induced conformational changes in the *Avena* photoreceptor suggests that light-dependent alterations in the exposure of regions near Ser_{598} and/or at the N-terminus are important to phytochrome-mediated signal transduction pathways. Experiments to address these possibilities, using transgenic plants containing mutagenized phytochrome cDNA constructs, are now feasible (Keller et al., 1989; Boylan & Quail, 1989; Kay et al., 1989b).

Phytochrome has been shown to be a phosphoprotein by in vivo labeling experiments (Quail et al., 1979) and by phosphate analysis on purified phytochrome preparations (Hunt & Pratt, 1980). These observations suggest a biological role for phytochrome phosphorylation in plants. It is significant to note that the copurifying, polycation-stimulated protein kinase phosphorylates the serine-rich N-terminus especially since a serine/threonine-rich region at the N-terminus of phytochrome appears to be conserved among all phytochromes whose primary structures have been deduced from cDNA sequence analyses (Hershey et al., 1985; Sharrock et al., 1988; Sato, 1988; Kay et al., 1989a; Sharrock & Quail, 1989). The importance of the N-terminus to the maintenance of native spectral properties of the photoreceptor has also been noted (Vierstra & Quail, 1986). The serine/threonine-rich sequence, found at the N-terminus of phytochrome, is unlike the consensus phosphorylation sequences for the major classes of protein kinases including the cyclic nucleotide dependent protein kinase, casein kinase, and tyrosine kinase enzyme families. The present studies confirm that three representatives of the mammalian serine/threonine class of protein kinases do not recognize this region of the phytochrome polypeptide. Experiments to address whether in vivo phosphorylations of phytochrome correspond to the same region of the photoreceptor which is phosphorylated by the polycation-stimulated protein kinase activity in vitro should shed new light on the biological significance of polycation-stimulated phytochrome phosphorylation.

Acknowledgments

We thank Richard A. Houghton, Scripps Clinic and Research Foundation, La Jolla, CA, for the gift of the synthetic peptides.

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Interactions of Retinol with Binding Proteins: Implications for the Mechanism of Uptake by Cells†

Noa Noy* and Zhi-Jun Xu
Department of Medicine, Cornell University Medical College, New York, New York 10021
Received August 22, 1989; Revised Manuscript Received October 27, 1989

ABSTRACT: The kinetic parameters of the interaction of retinol with retinol binding protein (RBP) were studied. The rate constant for association of retinol with the protein (k_a) was found to be 1.5×10^6 M^{-1} min^{-1}. The rate constant for dissociation (k_d) from the protein was determined by studying the transfer of retinol from RBP to lipid bilayers. It was found that such transfer proceeds via the aqueous phase and its rate-limiting step is the dissociation of retinol from the binding protein. The rate of transfer therefore represents the rate of dissociation. The k_d was 0.112 min^{-1}. These values were validated further by the following consideration. The equilibrium dissociation constant of RBP and retinol can be calculated from the expression $K_d = k_d/k_a$. The calculated value was 7.5×10^{-8} M. K_d was also measured directly by fluorometric titration and was found to be 7×10^{-8} M. The relative avidities of retinol for RBP, the complex RBP-transthyretin (RBP-TTR), and serum albumin were also studied. It was found that binding of RBP to TTR increased its avidity for retinol by about 2-fold. The avidity of albumin for retinol was 30-fold lower than that of RBP. The data imply that retinol spontaneously and rapidly dissociates from sites on binding proteins, which indicates that the vitamin can freely move in vivo between physiologic compartments with avidities for it.

Vitamin A alcohol is poorly soluble in water and is transported in blood bound to a protein complex that is constituted of retinol binding protein (RBP) and transthyretin (TTR) (Goodman & Blaner, 1984). The mechanism by which retinol moves from the protein complex in blood to the cytosol of target cells is not clear. This question has been studied by several laboratories, and it is usually proposed that specific receptors for RBP, mediating the uptake of retinol, exist in the plasma membranes of target cells (Chen & Heller, 1977; Rask & Peterson, 1976; McGuire et al., 1981; Heller, 1975; Ottonello & Maraini, 1981; Pfeffer et al., 1986). However, the existence of such receptors has not been demonstrated directly. The evidence given in support of putative receptors is the saturation of uptake of retinol by cells (Chen & Heller, 1977; Rask & Peterson, 1976) or the identification of proteins that bind RBP in plasma membranes (McGuire et al., 1981; Heller, 1975). Neither of these lines of evidence is sufficient

† Supported by American Cancer Society Grant BG-551. A part of this work was presented at the 32nd Annual Meeting of the Biophysical Society, Phoenix, AZ, Feb 1988.
* To whom correspondence should be addressed.

0006-2960/90/0429-3878$02.50/0