Partial Purification and Peptide Mapping of Ubiquitin–Phytochrome Conjugates from Oat†

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ABSTRACT: Phytochrome is rapidly degraded in vivo following photoconversion from the relatively stable red light absorbing form Pr to the far red light absorbing form Pfr. In etiolated seedlings from several species, this photoconversion also induces the accumulation of ubiquitin–phytochrome conjugates (Ub–P), suggesting that Pfr is degraded via a ubiquitin-dependent proteolytic pathway [Shanklin et al. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 359–363]. To understand why Pfr is preferentially conjugated with ubiquitin, Ub–P were partially purified and characterized with the ultimate goal of mapping ubiquitin attachment sites. Ub–P were partially purified by poly(ethylene imine) and ammonium sulfate precipitations followed by hydroxyapatite chromatography and separated from unmodified phytochrome by size-exclusion chromatography. Ub–P had an apparent native molecular size of approximately 600 kDa, substantially larger than that of the unmodified Pfr dimer (365 kDa). Ub–P retained the property of spectral photoreversibility as shown by their differential sensitivity to digestion with trypsin following irradiations with red or far-red light. The initial digestion patterns of Ub–P were similar to unmodified phytochrome, showing a more rapid cleavage following far-red irradiation, generating fragments 10 kDa smaller than intact Ub–P, and exhibiting a preferential loss of the N-terminus. In an attempt to identify ubiquitin attachment site(s), Ub–P were probed with a library of anti-oat phytochrome monoclonal antibodies. The ability of various monoclonal antibodies to immunoprecipitate tryptic fragments of Ub–P indicated that few, if any, ubiquitins were attached near the N-terminus of the chromoprotein and that a majority were attached to a C-terminal non-chromophore-containing domain. Digestion patterns of Ub–P by trypsin suggested that up to five ubiquitins may be attached to phytochrome at or near this domain. Of the 16 different anti-phytochrome monoclonal antibodies tested, 3 (O76C, O19F, and O311B) poorly recognized all size classes of Ub–P, indicating that the corresponding epitopes were masked either directly or indirectly as a result of ubiquitin ligation. These epitopes are located between residues 90 and 180 (O76C), 558 and 668 (O19F), and 747 and 830 (O311B) of oat phytochrome. Because the regions recognized by O19F and O311B are near the C-terminal domain containing at least one ubiquitin attachment site, and near amino acid residues that become more accessible when the chromoprotein is in the Pfr form, these regions may be important in the Pfr-dependent ubiquitination of phytochrome.

Many aspects of plant growth and development are controlled by the regulatory photoreceptor phytochrome [for a review, see Kendrick and Kronenberg (1986)]. In oat, phytochrome consists of a linear tetrapyrrole chromophore covalently linked to cysteine-321 of a 124-kilodalton (kDa)[1] protein (Hershey et al., 1985; Vierstra & Quail, 1986). It can exist in one of two photointerconvertible forms, a red light absorbing form, Pr, and a far red light absorbing form, Pfr. Conversion of Pr to Pfr involves a cis/trans isomerization of the chromophore as well as conformational changes at the N-terminus and within the apoprotein (Rüdiger, 1986; Largarias, 1986; Vierstra & Quail, 1986; Chai et al., 1987; Grimm et al., 1988a). Only Pfr appears to have biological activity, but its mode of action is currently unknown.

In plants exposed to red light, the levels of the active Pfr form are precisely controlled in part by (i) autoregulation of phytochrome gene transcription by Pfr (Colbert et al., 1983; Lissemore & Quail, 1988) and (ii) preferential breakdown of the chromoprotein, with Pfr degraded 50–100 times faster than Pr (Pratt, 1978). Recently, a potential mechanism for rapid Pfr degradation was proposed on the basis of the discovery that phytochrome from a variety of plant species becomes multiply conjugated with ubiquitin after Pfr formation (Shanklin et al., 1987; Jabben et al., 1989a). Ubiquitin is a 76 amino acid, eukaryotic polypeptide required for the degradation of abnormal and short-lived proteins in animals and yeast [for a review, see Finley and Varshavsky (1985) and Hershko (1988)]. Through a multienzyme system which requires ATP, ubiquitin functions by forming covalent intermediates with proteins targeted for degradation. Ligation occurs through an isopeptide bond between the C-terminal glycine carboxyl group of ubiquitin and free lysyl amino groups on the target protein. Once conjugated with one or more ubiquitins, the target protein is rapidly degraded by proteases specific for ubiquitin–protein conjugates, and free functional ubiquitin is released (Hough et al., 1986). Both the kinetics of ubiqui-

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[1] Abbreviations: Pr and Pfr, red light absorbing and far red light absorbing forms of phytochrome, respectively; Ub–P, ubiquitin–phytochrome conjugate(s); $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s).

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tin–phytochrome conjugate (Ub–P) accumulation and the localization of Ub–P during Pfr degradation are consistent with a proposal that Pfr is degraded by a ubiquitin-dependent proteolytic pathway (Shanklin et al., 1987; Jabben et al., 1989a,b). Ubiquitination of Pfr is of interest because it appears to represent the *first* example of a specific intracellular protein that is degraded by the ubiquitin system in vivo and the *first* posttranslational modification reported for phytochrome that occurs in vivo and is specific for the active form.

Selectivity of the ubiquitin system appears to reside in the specific recognition of target proteins by enzymes involved in ubiquitin conjugation (Hershko, 1988). To understand why Pfr is preferentially recognized, we have begun to physicochemically characterize Ub–P. Our ultimate goal is to identify site(s) for ubiquitin attachment and explore the possibility that such site(s) is (are) more accessible on Pfr than on Pr. Here, we describe the partial purification and initial characterization of oat Ub–P. Using a variety of immunological techniques in conjunction with anti-phytochrome monoclonal antibodies, we identified at least one site for ubiquitin ligation.

Materials and Methods

Plant Materials. Oat seedlings (*Avena sativa* [L.] cv. Garry) were grown in open containers in darkness for 4.5 days at 25 °C. Seedlings were irradiated with red light for 10 min using a Sylvania Gro Lux F40/GRO light source (I = 3 W/m^2) that converts 86% of Pr to Pfr. Following the irradiation, seedlings were incubated in darkness for 80 min at 25 °C before being harvested. All subsequent manipulations were performed at 0–4 °C under dim green safelight unless otherwise specified.

Purification of Ub–P. Phytochrome and Ub–P were partially purified from the apical 3–4 cm of oat shoots according to the method of Vierstra and Quail (1983). Briefly, shoots were homogenized in 50% (v/v) ethylene glycol, 100 mM Tris-HCl, 140 mM ammonium sulfate, 10 mM Na_4EDTA, 20 mM sodium metabisulfite, and 4 mM PMSF, pH 8.3 (4 °C). The homogenate was made 0.1% (w/v) in poly(ethylene imine) and clarified by centrifugation at 14000g. Proteins that precipitated following the addition 250 g/L ammonium sulfate were resuspended in 25% (v/v) ethylene glycol, 50 mM Tris-HCl, 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF (pH 7.8, 4 °C) and applied to a (2.5 × 10 cm) hydroxyapatite column equilibrated in the same buffer with the addition of 140 mM ammonium sulfate. The column was washed with 2 column volumes of 50 mM Tris-HCl, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol (pH 7.8) followed by 4 column volumes of 5 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol (pH 7.8). Phytochrome and Ub–P were eluted with 20 mM potassium phosphate, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol (pH 7.8). The hydroxyapatite eluant was concentrated to approximately 1 mg/mL phytochrome by ammonium sulfate precipitation, irradiated with saturating red light, and applied to a 2.5 × 100 cm Bio-Gel A-1.5 M size-exclusion column (Bio-Rad) equilibrated with 50 mM Tris-HCl, 5 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, pH 7.8 (4 °C). Fractions containing high levels of ubiquitin-immunoreactive species and low levels of unmodified phytochrome (as determined spectrophotometrically and by immunoblotting) were pooled and concentrated 10-fold by ammonium sulfate precipitation. This pool was resuspended in 50 mM Tris-HCl and 5 mM Na_4EDTA, pH 7.8 (4 °C), and was used for experiments involving Ub–P. The Bio-Gel A-1.5M column was calibrated with respect to molecular size using the following proteins: thyroglobulin, 660 kDa; oat phytochrome as Pfr, 365 kDa; catalase, 232 kDa; alcohol dehydrogenase, 150 kDa; carbonic anhydrase, 29 kDa; cytochrome c, 12.4 kDa (Maire et al., 1980; Lagarias & Mercurio, 1985).

Antibody Preparation. Polyclonal antibodies against purified 124-kDa oat phytochrome were prepared as previously described (Shanklin et al., 1987). Monoclonal antibodies against oat phytochrome either were those described by Daniels and Quail (1984) or were raised against purified (A_{660}/A_{280} greater than 0.9) oat phytochrome by using the procedure of Köhler and Milstein (1975), with the modifications of Mierendorf and Dimond (1983) and Tyre and Pate (1988). In this study, phytochrome was denatured by boiling for 3 min in 1% (w/v) $NaDodSO_4$ before injection into Balb/cAu mice. The primary screen of NS/1 myeloma cell fusions for anti-phytochrome antibodies was by direct ELISA (Cordonnier et al., 1984). Monoclonal antibodies were produced in ascites tumors and purified from the fluid by protein A affinity chromatography (Ey et al., 1978). Approximate locations of phytochrome epitopes recognized by the monoclonal antibodies were mapped by proteolytic fragmentation of the chromoprotein followed by immunoblot analysis (Daniels & Quail, 1984; Jones & Quail, 1989). For several monoclonals, these locations were confirmed by immunoblot analysis of lacZ–phytochrome fusion proteins generated by inserting various deletion fragments of a phytochrome-encoding cDNA into λgt11 (Thompson et al., 1987).

Immunological Techniques. Immunoprecipitation of native phytochrome was performed as previously described (Shanklin et al., 1987) using mouse anti-phytochrome monoclonal antibodies (either in ascites fluid or protein A purified) in conjunction with *Staphylococcus aureus* cells. When monoclonal antibodies that recognized denatured phytochrome were used, Ub–P-containing samples were first boiled for 2 min in 1% (w/v) $NaDodSO_4$ and diluted into 8 volumes of 50 mM Tris-HCl and 1% (w/v) Triton X-100, pH 7.8 (25 °C). This solution was clarified at 14000g for 5 min and the supernatant used for immunoprecipitation as described above. Discontinuous $NaDodSO_4$–PAGE was performed according to Laemmli (1970) using an acrylamide:bis(acrylamide) ratio of 30:0.8. Proteins were transferred onto nitrocellulose and subjected to immunoblot analysis as described (Jabben et al., 1989b). Immunoreactive bands were visualized colorimetrically by using alkaline phosphatase conjugated goat anti-rabbit or anti-mouse antibodies and the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Tryptic Digestions. Ub–P pool was made 50 mM Tris-HCl, pH 7.8 (22 °C), and irradiated with red light (I = 35 W/m^2), far-red light (I = 14 W/m^2), or red followed by far-red light. Trypsin (Sigma type XI) was added at a 1:500 (w/w) trypsin:total protein ratio, and the digestion mixtures were incubated at 22 °C in darkness. At various times, aliquots were boiled for 2 min in an equal volume of $NaDodSO_4$–PAGE sample buffer (Shanklin et al., 1987), cooled to 0 °C, and mixed with a 5-fold excess (w/w) of soybean trypsin inhibitor. The resulting digests were analyzed by immunoblot analysis as described above.

Results

Purification of Ub–P. Ub–P was partially purified from etiolated oat shoots irradiated with red light by using a modification of the procedure of Vierstra and Quail (1983) developed for the isolation of 124-kDa oat phytochrome. Ub–P and phytochrome copurified during poly(ethylene imine) and ammonium sulfate fractionations and hydroxyapatite chromatography. Then conjugates were separated from the bulk of phytochrome by size-exclusion chromatography performed

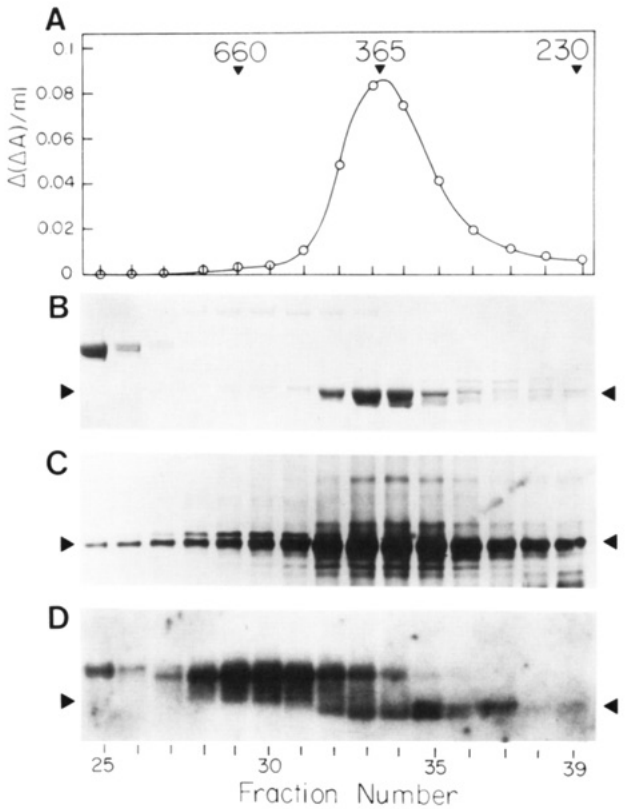

FIGURE 1: Size-exclusion chromatography of Ub–P. A hydroxyapatite pool containing both phytochrome and Ub–P was loaded onto a 2.5 cm × 100 cm Bio-Gel A-1.5 M size-exclusion column. Fractions (5 mL) were collected and analyzed for protein, phytochrome, and Ub–P. (Panel A) Photoreversible phytochrome content of various fractions as determined by dual-wavelength (A_{666}–A_{730}) difference spectroscopy [$\Delta(\Delta A)$]. (Panel B) Protein profile as determined by $NaDodSO_4$–PAGE and Coomassie staining. (Panel C) Phytochrome content as determined by $NaDodSO_4$–PAGE and immunoblot analysis with anti-phytochrome antibodies. (Panel D) Ub–P content as determined by $NaDodSO_4$–PAGE and immunoblot analysis with anti-ubiquitin antibodies. Equal volumes from each fraction were subject to $NaDodSO_4$–PAGE on 8% acrylamide gels. Elution peaks of the various protein standards used to calibrate the Bio-Gel column are indicated by arrowheads in panel A. The $NaDodSO_4$–PAGE mobility of 124-kDa phytochrome is indicated by arrowheads in panels B, C, and D.

under nondenaturing conditions (Figure 1). Here, a majority of Ub–P eluted from a Bio-Gel A-1.5M column with a broad profile, well ahead of the unmodified phytochrome dimer, whose native size as Pfr is 365 kDa (Lagarias & Mercurio, 1985). On the basis of their elution from the Bio-Gel column, the native molecular size of Ub–P was estimated to be between 350 and 1000 kDa, with a peak at ~600 kDa (Figure 1). A small portion of Ub–P coeluted with 124-kDa phytochrome. On the basis of $NaDodSO_4$–PAGE mobility (Figure 1D), these species appeared to represent proteolytic breakdown products of Ub–P and were discarded. By combination of fractions preceding phytochrome, a conjugate pool containing greater than 50% of the immunodetectable Ub–P and less than 10% of the spectrally detectable phytochrome was obtained.

Ub–P purified in the manner above had apparent molecular masses between 129 and 170 kDa as determined by $NaDodSO_4$–PAGE and were the sole ubiquitin-immunoreactive species in the conjugate pool (see Figure 3, $t = 0$). However, they still represented only a small percentage of the total protein despite an approximately 200-fold purification (based on immunoblot and protein assays), with unmodified phytochrome being a major contaminant. The Ub–P in these preparations were weakly visible in nickel-stained $NaDodSO_4$–PAGE gels (Kodavue, Eastman Kodak Co.) but were easily observed by immunoblotting with anti-ubiquitin antibodies.

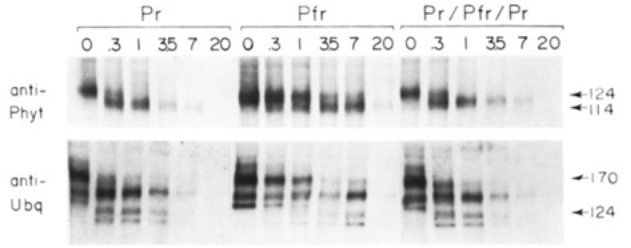

FIGURE 2: Tryptic digestion of Ub–P and phytochrome after various light treatments. Partially purified Ub–P, containing both Ub–P and unmodified phytochrome, were irradiated either with far-red light (Pr), with red light (Pfr), or with red followed by far-red light (Pr/Pfr/Pr). Trypsin was added, and the digestion mixtures were incubated at 22 °C in darkness (see Materials and Methods). At the times indicated (in hours), aliquots were removed and subjected to $NaDodSO_4$–PAGE [using an 8% acrylamide gel (upper panel) or a 6% acrylamide gel (lower panel)] followed by immunoblot analysis with either anti-phytochrome antibodies (upper panel) or anti-ubiquitin antibodies (lower panel). Each lane contained an equal volume of the digestion mixture, corresponding to an initial phytochrome content of 0.15 μg (upper panel) or 1.5 μg (lower panel). The apparent molecular masses (in kilodaltons) of the various phytochrome polypeptides are indicated by arrows.

Attempts to purify Ub–P further met with limited success. These included AffiGel Blue chromatography of the hydroxyapatite pool (Vierstra & Quail, 1983) and the isolation of Mg^{2+}-induced pelletable phytochrome as a first step in the purification (Pratt & Marmé, 1976). The latter strategy was based on the observation that Ub–P preferentially fractionates with pelletable phytochrome (Jabben et al., 1989b).

Photoreversibility of Ub–P. After partial purification, Ub–P were still too low in abundance and contaminated with unmodified phytochrome to measure their spectral characteristics by conventional methods. However, the differential sensitivity of Pr and Pfr to trypsin (Largarias & Mercurio, 1985) was exploited to determine if the phytochrome moiety in Ub–P retained a photoreversible chromophore. As previously described (Lagarias & Mercurio, 1985; Grimm et al., 1986, 1988a), trypsin initially cleaved unmodified phytochrome into a 114-kDa chromopeptide intermediate with Pr cleaved faster than Pfr (Figure 2, upper panel). When Ub–P were incubated with trypsin, an irradiation-dependent digestion of the conjugates was also observed (Figure 2, lower panel). This digestion was similar to unmodified phytochrome, occurring more rapidly (>10×) in preparations irradiated with far-red light (Pr) and red light followed by far-red light (Pr → Pfr → Pr) than in preparations irradiated with red light (Pfr). Each size class of Ub–P appeared to lose approximately 10 kDa, which was similar to the 10 kDa initially cleaved from unmodified phytochrome.

The initial 10-kDa cleavage of unmodified phytochrome by trypsin involves a simultaneous loss of approximately 7 kDa from the N-terminus and approximately 4 kDa from the C-terminus (Grimm et al., 1986). Proof that the 10-kDa tryptic cleavage of Ub–P also affected the N-terminus of phytochrome was provided by immunoprecipitation of tryptic fragments with anti-phytochrome monoclonal antibodies (Figure 3). All cleavage fragments of Ub–P and unmodified phytochrome were immunoprecipitated by a monoclonal antibody directed against a phytochrome domain located approximately between amino acids 90 and 180 [type II (Daniels & Quail, 1984; Jones & Quail, 1989)] (Figure 3). In contrast, a monoclonal antibody that recognizes a domain within 33

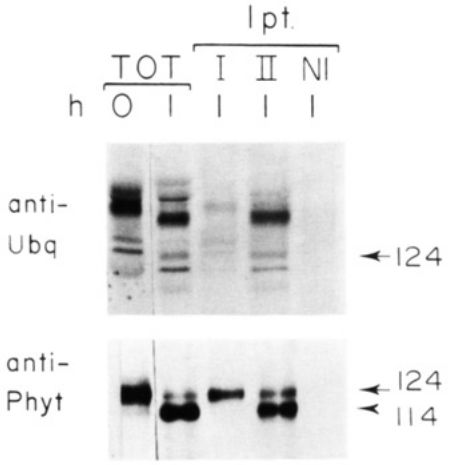

FIGURE 3: Location of the initial 10-kDa tryptic cleavage site of phytochrome and Ub–P. Ub–P and phytochrome were irradiated with far-red light and digested with trypsin for 1 h in darkness. The digestion products were immunprecipitated using type I (I) and II (II) anti-phytochrome and nonimmune (NI) monoclonal antibodies according to Materials and Methods. Immunoprecipitates were subjected to $NaDodSO_4$–PAGE and immunoblot analysis with either anti-ubiquitin antibodies (upper panel) or anti-phytochrome antibodies (lower panel). Each lane contained an equal volume of the digestion mixture corresponding to an initial phytochrome content of 1.5 μg (upper panel) or 0.15 μg (lower panel). The apparent molecular masses (in kilodaltons) of the various phytochrome polypeptides are indicated by arrows. TOT = digestion samples before immunoprecipitation. Ipt = immunoprecipitates.

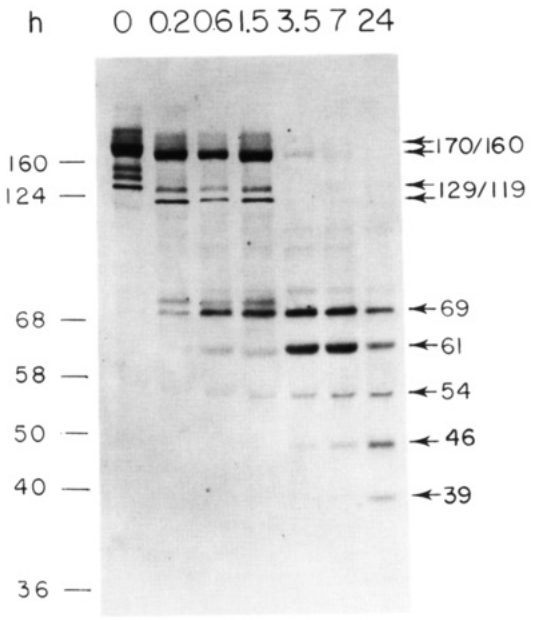

FIGURE 4: Tryptic digestion pattern of Ub–P after irradiation with far-red light. Partially purified Ub–P were irradiated with far-red light and incubated with trypsin at 22 °C in darkness. At the time indicated, aliquots were removed and subjected to $NaDodSO_4$–PAGE using an 8% acrylamide gel and immunoblot analysis with anti-ubiquitin antibodies. Each lane contained an equal volume of digestion mixture corresponding to an initial phytochrome content of 1.5 μg. The apparent molecular masses (in kilodaltons) of predominant ubiquitin-containing polypeptides are indicated by arrows.

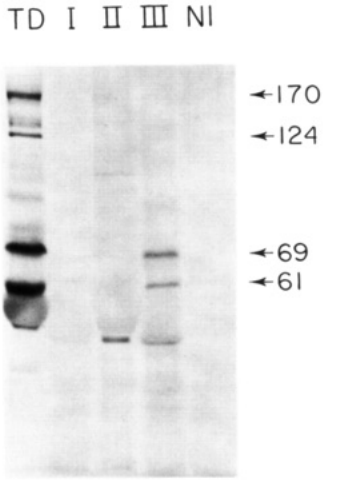

FIGURE 5: Immunoprecipitation of Ub–P tryptic peptides with anti-phytochrome monoclonal antibodies. Partially purified Ub–P were irradiated with red light and digested with trypsin for 7 h in darkness. Phytochrome-containing polypeptides were immunoprecipitated with either types I, II, and III anti-phytochrome antibodies or nonimmune (NI) monoclonal antibodies under denaturing conditions (see Materials and Methods). Immunoprecipitates were subjected to $NaDodSO_4$–PAGE on an 8% acrylamide gel and immunoblot analysis with anti-ubiquitin antibodies. The apparent molecular masses (in kilodaltons) of the various ubiquitin-containing polypeptides are indicated by arrows. TD = digestion mixture before immunoprecipitation.

amino acids of the N-terminus [type I (Daniels & Quail, 1984; Grimm et al., 1986)] failed to recognize 114-kDa phytochrome and all cleavage fragments of Ub–P. This antibody still was able to recognize residual amounts of undigested Ub–P and phytochrome (Figure 3).

Mapping of Ubiquitin Attachment Sites. The observation that removing approximately 10 kDa from Ub–P altered only the molecular mass, and not the profile of ubiquitinated species (Figures 2 and 3), indicated that the majority of ubiquitin was not attached within 7 kDa of the N-terminus or 4 kDa of the C-terminus of phytochrome. Attempts to define ubiquitin attachment sites further involved both peptide mapping with trypsin and immunorecognition studies with a library of anti-phytochrome monoclonal antibodies. The monoclonal antibodies used either were generated by us against $NaDodSO_4$-denatured 124-kDa oat phytochrome or were those described by Daniels and Quail (1984), prepared against native 124-kDa oat phytochrome [types I, II, and III (see Figure 7)]. Trypsin was chosen because while phytochrome is rapidly digested into an array of peptides by this protease (Laragias & Mercurio, 1985), the ubiquitin moiety of conjugates is relatively resistant (Schlesinger et al., 1975; Vierstra et al., 1986). (Under nondenaturing conditions, only the C-terminal Gly-Gly of oat ubiquitin is susceptible to trypsin cleavage, with strong denaturing conditions required for complete digestion of the molecule.)

While brief digestions of Ub–P with trypsin resulted in a series of digestion products all missing 10 kDa, prolonged digestions produced two prominent ubiquitinated species at 69 and 61 kDa with the 69-kDa species appearing first (Figure 4). These two species appeared to contain the majority of the ubiquitin in the Ub–P preparations. The 69- and 61-kDa polypeptides were gradually degraded to smaller species, generating a series of five ubiquitin-containing polypeptides from 69 to 39 kDa. In an effort to map the 69- and 61-kDa species, digestion mixtures containing these polypeptides were immunoprecipitated with monoclonal antibodies specific for the type I, II, and III domains of unmodified phytochrome (Daniels & Quail, 1984). Each monoclonal recognized Ub–P prior to trypsin digestion. Of the three tested, only a type III antibody, specific for a phytochrome domain approximately between amino acids 790 and 830, immunoprecipitated the 69- and 61-kDa species (Figure 5). A lower molecular mass species was also found in immunoprecipitates with the type II and III antibodies. However, because this species was not

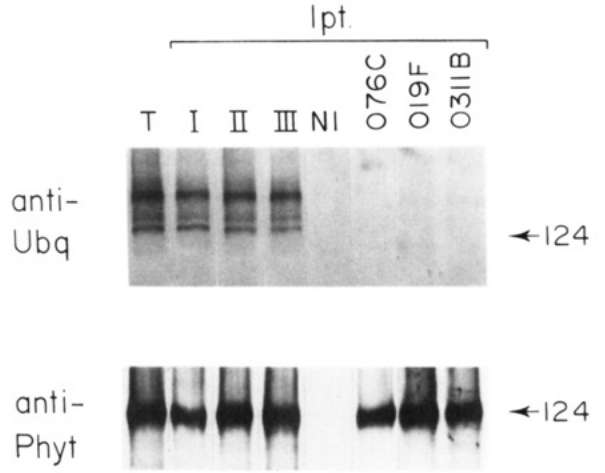

FIGURE 6: Recognition of Ub–P by various anti-phytochrome monoclonal antibodies. Ascites fluids containing various anti-phytochrome monoclonal antibodies were incubated with partially purified Ub–P under denaturing (O76C) or native (types I, II, III, NI, O19F, and O311B) conditions. Locations of the phytochrome domains recognized by the monoclonal antibodies are indicated in Figure 7. Phytochrome polypeptides were immunoprecipitated and subjected to $NaDodSO_4$–PAGE using an 8% acrylamide gel and immunoblot analysis with anti-ubiquitin antibodies (upper panel) or anti-phytochrome antibodies (lower panel). The samples used for the various immunoprecipitations contained either 1 μg (upper panel) or 0.1 μg (lower panel) of phytochrome. Equal volumes of the immunoprecipitates were applied to each gel lane. Lane T contains an aliquot of the Ub–P preparation before immunoprecipitation. NI = nonimmune monoclonal antibody. The position of the 124-kDa phytochrome monomer is shown on the right.

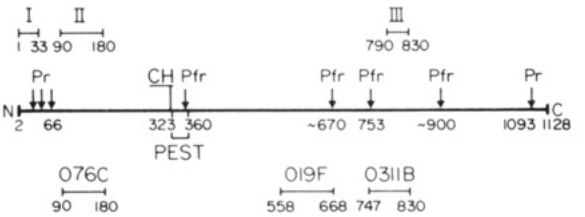

FIGURE 7: Structural map of oat phytochrome showing the chromophore attachment site (CH), the PEST sequence (Rogers et al., 1986), several form-dependent protease cleavage sites preferential for either the Pr or the Pfr forms (Vierstra et al., 1984; Lagarias & Mercurio, 1985; Grimm et al., 1988a), and regions containing epitopes recognized by the various anti-phytochrome monoclonal antibodies used here (I, II, III, O76C, O19F, and O311B). Numbers indicate the positions of the amino acid residues that define each phytochrome domain. The locations of the type II and III epitopes and the protease cleavage sites at positions 670 and 900 are approximate values.

observed in the total digest before immunoprecipitation, the origin of this species is uncertain.

Immunorecognition of Ub–P with Monoclonal Antibodies. Given the relatively large size of ubiquitin, it is possible that ubiquitin ligation substantially alters access to the surface of phytochrome. Ub–P were examined for such alterations by testing an array of anti-phytochrome monoclonal antibodies for their ability to recognize Ub–P. The monoclonal lines tested included four that recognized epitopes within the type I domain, eight that recognized epitopes within the type II domain, two that recognized epitopes within the type III domain, and two that recognized epitopes between the type II and III domains (see Figure 7).

Of the 16 different monoclonal antibodies tested, all recognized unmodified phytochrome present in the partially purified Ub–P preparations (Figure 6, lower panel, data not shown). When analyzed for Ub–P, 13 of the 16 also effectively recognized Ub–P, with all size classes of Ub–P (129–170 kDa) present in the immunoprecipitates (Figure 6, upper panel, data not shown). However, three monoclonal antibodies (O76C, O19F, and O311B) showed substantially reduced immunorecognition (<20%) of Ub–P. These antibodies failed to immunoprecipitate every size class of Ub–P, suggesting that the corresponding epitopes for these antibodies were altered in a similar fashion. Monoclonal O76C, which binds only to $NaDodSO_4$-denatured phytochrome, failed to recognize Ub–P denatured under similar conditions. Monoclonal O19F, which binds to both native and denatured phytochrome, failed to recognize Ub–P under native conditions but recognized all classes of Ub–P following $NaDodSO_4$ denaturation. Monoclonal O311B, which also binds to both native and denatured phytochrome, failed to recognize both native and denatured Ub–P (Figure 6 and data not shown).

The approximate locations of the epitopes for monoclonal antibodies, O76C, O19F, and O311B, were identified by conventional peptide mapping (Daniels & Quail, 1984; Jones & Quail, 1989). The locations of the epitopes for O19F and O311B were more precisely defined by immunorecognition of phytochrome cDNA deletion mutants expressed in λgt11 (Thompson et al., 1987). Monoclonal antibody O76C recognized an epitope within the type II domain (approximately between amino acid residues 90 and 180), O19F recognized an epitope between residues 558 and 668, and O311B recognized an epitope between residues 747 and 830 (data not shown). The relationships of these epitopes with other domains on phytochrome are shown in Figure 7.

Discussion

In this study, we describe the purification and initial characterization of Ub–P synthesized in vivo following the irradiation of etiolated oat seedlings with red light. Whereas extracts from red light irradiated plants accumulate a heterogeneous population of conjugates with molecular masses exceeding 200 kDa (Shanklin et al., 1987), only a subset of this population enriched for discrete size classes of Ub–P from 129 to 170 kDa was present in the final purified preparations. It is possible that these species represent a stable subset of total Ub–P. The apparent linear relationship between molecular mass and the number of ubiquitins attached for model substrates (Hershko et al., 1980) indicates that this preparation of Ub–P contains from one to seven ubiquitins per phytochrome monomer.

Conjugation of ubiquitin to phytochrome does not appear to substantially alter the phytochrome moiety as judged by the retention of red/far-red photoreversibility and the similarity of initial tryptic cleavage patterns. However, the possibility that ubiquitin conjugation interferes with the conformation and/or spectral properties of Pr and Pfr in a more subtle way cannot be discounted. Differences between phytochrome and Ub–P that were detected involved alterations in the shape and/or surface of the chromoprotein. These differences are most easily explained by the presence of ubiquitin moieties. The pronounced increase in the native size of phytochrome after ubiquitin attachment, especially for the 129-kDa species of Ub–P which likely possesses only a single ubiquitin moiety, suggests that the linked ubiquitins extend significantly from the chromoprotein's surface. Of interest is the observation that all size classes of Ub–P, from 129 to 170 kDa, eluted with approximately the same profile despite differences in the presumed number of attached ubiquitins (Figure 1). Whether both monomers of the phytochrome dimer contain ubiquitin moieties is unknown.

On the basis of our studies, the location of at least some ubiquitin attachment sites on Ub–P can be proposed. Little

if any ubiquitin appears to be attached within 7 kDa of the N-terminus or 4 kDa of the C-terminus based on the tryptic cleavage studies (Figures 2 and 3). An unmodified N-terminal region is supported also by the facts that the N-terminal residue (serine) is blocked (Vierstra & Quail, 1983; Grimm et al., 1988b) and that the first lysine does not appear until residue 46 (Hershey et al., 1985). Most of the ubiquitin-immunoreactive signal resides in tryptic fragments of 69 and 61 kDa. These fragments can be assigned to a region approximately two-thirds of the way from the N-terminus based on the polypeptides' immunorecognition with a type III monoclonal antibody (Figures 5 and 7) and can extend from the type III epitope toward the C-terminus and/or toward the N-terminus. The association of most ubiquitins with the 69- and 61-kDa fragments indicates that ubiquitins are not randomly conjugated to many different sites along the chromoprotein but that their attachment is concentrated to specific region(s).

The kinetics of trypsin digestion are consistent with the possibility that the 69-kDa polypeptide represents a digestion intermediate of the 170-kDa species which then is processively cleaved into an array of lower molecular mass species of 61, 54, 46, and 39 kDa (Figure 5). The fact that each one of these species differs in molecular mass by approximately the size of a ubiquitin monomer (8.5 kDa) suggests that the ladder of ubiquitin peptides could result from a slow cleavage of five ubiquitin moieties from a single phytochrome peptide. However, the more remote possibility that this ladder represents four incremental cleavages of 7–8 kDa from the phytochrome moiety cannot be ruled out. If the 69-kDa species is a ubiquitinated polypeptide containing five or more ubiquitins, then a core phytochrome peptide with a mass no greater than 32 kDa would be predicted. The five ubiquitins could be attached directly to individual lysines in this peptide or indirectly attached to a single lysine through a branched ubiquitin structure such as that reported by Hershko and Heller (1985).

The reduced recognition of Ub–P by three anti-phytochrome monoclonal antibodies (O76C, O19F, and O311B) identifies additional phytochrome domains affected by ubiquitin conjugation (Figures 6 and 7). The fact that the recognition of all size classes of Ub–P was attenuated suggests that the corresponding epitopes on each size class of Ub–P were altered in a similar way. All three monoclonals bind to NaDodSO$_4$-denatured phytochrome and phytochrome immobilized on nitrocellulose, indicating that they recognize primary sequence epitopes. Because both monoclonals O76C and O311B showed reduced recognition of NaDodSO$_4$-denatured Ub–P, it is likely that domains surrounding the corresponding epitopes are directly altered in Ub–P. Whether this results from attachment of ubiquitin(s) at or near the corresponding epitopes, or from another as yet unknown modification associated with conjugate formation, is not yet clear. Certainly, the proximity of the O311B epitope to a phytochrome domain containing ubiquitin attachment site(s) [type III (Figure 5)] suggests that this epitope may be directly blocked by ubiquitin attachment. While monoclonal O19F was unable to recognize Ub–P in its native state, it could recognize NaDodSO$_4$-denatured Ub–P. Thus, it is likely that the corresponding epitope for O19F is not directly altered in Ub–P but may be sterically hindered in the native molecule by ubiquitins attached at adjacent domains.

Antibodies O19F and O311B deserve special attention because they recognize phytochrome epitopes that are within or close to the 69- and 61-kDa fragments containing most of the attached ubiquitin following trypsin digestion. Furthermore, these epitopes are near phytochrome residues ~670, 753, and ~900 that become more susceptible to protease digestion following photoconversion of Pr to Pfr (Vierstra et al., 1984; Lagarias & Mercurio, 1985; Grimm et al., 1988a) (see Figure 7). Thus, it is possible that domains within this region become more accessible as Pfr and are then recognized by the ubiquitin conjugation system, and subsequently modified with one or more ubiquitins.

Assuming that Pfr is degraded via a ubiquitin-dependent proteolytic pathway, phytochrome may provide relevant information with respect to two current models for selective protein breakdown. The "N-End Rule" of Varshavsky and co-workers states that the degradation rate of a protein is determined by the nature of its N-terminal residue (Bachmair et al., 1986). As a mechanism, they proposed that N-terminal residue of a target protein is recognized by a component of the ubiquitin system followed by a processive ubiquitination of lysyl residues from the N-terminus to the C-terminus before the protein is degraded. On the basis of available data, Pfr-specific phytochrome degradation does not obey this rule. The N-terminal residue (serine) of phytochrome purified either as Pr or as Pfr is blocked (Vierstra & Quail, 1983; Litts et al., 1983; Grimm et al., 1988b), which should stabilize the chromoprotein according to the N-End Rule. Furthermore, approximately 10 kDa of the N-terminus of phytochrome undergoes a substantial conformational change following photoconversion of Pr and Pfr, but the region is *less* exposed in Pfr (Lagarias, 1985; Vierstra & Quail, 1986). We find little if any ubiquitin conjugation at or near the N-terminus and no evidence for processive attachment, and observe that only a few specific sites within the primary sequence of phytochrome may be ubiquitinated (Jabben et al., 1989b; this report). However, it is possible that these attachment sites lie adjacent to the N-terminus in the native chromoprotein.

With respect to the "PEST" hypothesis of Rechsteiner and co-workers (Rogers et al., 1986), a PEST sequence has been identified in phytochrome between residues 323 and 360 and adjacent to the chromophore (Hershey et al., 1985) (Figure 7). Upon photoconversion from Pr to Pfr, phytochrome's chromophore undergoes a cis–trans isomerization (Rüdiger, 1986) and a 31° reorientation with respect to the apoprotein (Eklund et al., 1985). This reorientation does expose residues within the PEST sequence, making them more accessible to proteases (Grimm et al., 1988a). Thus, it is possible that phototransformation of Pr to Pfr could expose the PEST sequence, allowing the sequence to be recognized by the ubiquitin conjugation system. However, our mapping studies argue against the PEST sequence as a site of direct ubiquitin attachment.

It is possible that specific ubiquitination of phytochrome involves neither the N-terminus nor the PEST domain. Following photoconversion of Pr and Pfr, the greater accessibility of amino acid residues near the epitope for O311B (747–830) may promote recognition of Pfr by the ubiquitin conjugating system. One or more ubiquitins could be attached to one or a few lysines near this epitope, blocking recognition by both monoclonal antibodies O19F and O311B. Clearly, identification of specific lysyl residues involved in ubiquitin ligation will help locate the important domains involved in Pfr-specific ubiquitination and degradation of phytochrome.

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