The plant photoreceptor chromoprotein, phytochrome, is rapidly degraded in vivo after photoconversion from a stable red light-absorbing form (Pr) to a far-red light-absorbing form (Pfr). Previously, we demonstrated that during Pfr degradation in etiolated oat seedlings, ubiquitin-phytochrome conjugates, (Ub-P), appear and disappear suggesting that phytochrome is degraded via a ubiquitin-dependent proteolytic pathway (Shanklin, J., Jabben, M., and Vierstra, R. D. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 359-363). Here, we provide additional kinetic and localization data consistent with this hypothesis by exploiting the unique ability to photoregulate phytochrome degradation in vivo. An assay for the quantitation of Ub-P was developed involving immunoprecipitation of total conjugates with anti-ubiquitin antibodies, followed by the detection of Ub-P with anti-phytochrome antibodies. Using this immunassay, we found that Ub-P will accumulate to approximately 5% of initial phytochrome during Pfr degradation induced by a saturating red light pulse. Reducing the amount of Pfr produced initially by attenuating the red light pulse, lowered the amount of phytochrome degraded in the following dark period and concomitantly reduced the maximal accumulation of Ub-P. Continuous far-red irradiations that maintained only 4% of phytochrome as Pfr induced rapid phytochrome degradation similar to that induced by a red light pulse converting 86% of Pr to Pfr. The appearance and disappearance of Ub-P were similar for each irradiation indicating that Ub-P accumulation is independent of the level of Pfr provided rapid phytochrome degradation is maintained. Pulse-chase studies employing continuous far-red light followed by darkness showed that Ub-P are continuously synthesized during phytochrome degradation and rapidly disappear once degradation ceases. Ub-P also accumulated during “cycled Pr” degradation induced by the transformation of Pr to Pfr and back to Pr. The commitment to degrade cycled Pr and form Ub-P occurred within seconds after Pfr formation making the cause(s) underlying this phenomenon one of the fastest phytochrome reactions known. Within seconds after Pfr formation, a majority of phytochrome is also known to aggregate in vitro (previously defined as sequestered or pelletable), with aggregated phytochrome preferentially lost during phytochrome degradation. In vitro analysis of aggregated phytochrome indicated that they contain most of the Ub-P. Moreover, the appearance of Ub-P in the aggregated and soluble fractions correlated with the time that phytochrome disappeared from that fraction. Taken together, these observations suggest that light-induced phytochrome degradation involves a step after Pfr formation that can rapidly commit phytochrome to degradation regardless of the final spectral form. This event is followed by conjugation of ubiquitin to the chromoprotein and finally catabolism of the conjugated protein. Both kinetic and localization data suggest that light-induced phytochrome aggregation represents an early step in degrading this chromoprotein.

An important characteristic of intracellular protein degradation is its selectivity (1). This selectivity is essential for regulating cell physiology, allowing proteins to coexist within the same cellular milieu with half-lives ranging from 10 min to greater than 100 h. How proteins are specifically chosen for degradation is unknown, although several mechanisms have been proposed recently, including recognition of the N-terminal residue (2, 3) or polypeptide domains rich in specific amino acids (4).

In eukaryotes, selective protein degradation is accomplished in part by a proteolytic pathway involving the 76-amino acid protein, ubiquitin (for reviews, see Refs. 5 and 6). The pathway appears responsible for degrading both abnormal proteins and short-lived proteins (5, 6), the latter includes many important regulatory proteins. In this pathway, ubiquitin becomes covalently linked to proteins destined for catabolism and serves as a reusable recognition signal for selective proteolysis. Ligation is accomplished by an ATP-coupled series of reactions involving at least three proteins (6) and occurs through the formation of an isopeptide bond between the C terminus of ubiquitin and free amino groups on the target protein. Once conjugated with one or more ubiquitins, target proteins are degraded by protease(s) that specifically recognize this modification and free ubiquitin is released (7). Alternatively, unmodified target proteins may be regenerated by isopeptidases that cleave only the isopeptide bond liberating both the target protein and ubiquitin intact (8, 9). The function of this disassembly reaction remains obscure.

Although eukaryotes contain a wide variety of ubiquitin conjugates (10, 11), only a few ubiquitinated species have been analyzed in detail (6). As a result, limited information is currently available concerning how specific conjugates are synthesized and degraded as well as how proteins are selectively recognized by the ubiquitin conjugation system. Moreover, while numerous studies indicate a role of ubiquitin in protein catabolism (6, 6), specific intracellular proteins that...
are actually degraded by the pathway have not yet been identified.

To understand the mechanism(s) responsible for selective protein degradation, we have begun to study the degradation of phytochrome as a model system. Phytochrome is a 124-kDa cytoplasmic chromoprotein, which exists in plants in two photoconvertible forms, a red-light-absorbing form, Pr, and a far-red-light-absorbing form, Pfr (for reviews, see Ref. 12). It is synthesized as Pr which has an in vivo half-life of >100 h (13). Upon photoconversion to Pfr, the degradation rate increases approximately 100-fold with half of the protein lost in 1-2 h (13–16). The ability to manipulate both synchronously and noninvasively the degradation rate of phytochrome can be exploited to study how the proteolytic machinery selects and degrades specific intracellular proteins while leaving others alone. In addition, because Pfr rapidly inhibits further synthesis of phytochrome mRNA in oat (17), it is possible to interpret kinetic data of Pfr catabolism without the interference of continued phytochrome synthesis.

Previously we demonstrated that following photoconversion of Pr to Pfr by a red light pulse, ubiquitin-phytochrome conjugates, Ub-P, are synthesized in etiolated oat seedlings (18). Because ubiquitin conjugation is involved in the degradation of many short-lived cytoplasmic proteins and because Ub-P diminishes during the course of phytochrome degradation, we postulated that phytochrome is degraded via the ubiquitin-dependent proteolytic pathway. However, when considering other possible functions of ubiquitin conjugation (18–21), alternative interpretations of the data are feasible. In the experiments described here, we provide an estimate for the pool size of Ub-P and describe their dynamics during phytochrome degradation. We also show that the commitment to form Ub-P occurs within seconds after Pfr formation and may involve aggregation of the photoreceptor. The data provide the first detailed description of the pool dynamics of a ubiquitin-protein conjugate and are consistent with the hypothesis that phytochrome is rapidly degraded via ubiquitin-activated intermediates.

EXPERIMENTAL PROCEDURES

Plant Material—Oat seeds (Avena sativa (L.) cv. Garry) were obtained from UW Foundation Seeds (Madison, WI). In experiments involving cycloheximide treatment, oat seeds provided as a gift by Dr. A. Mancinelli (Columbia University, New York) were used (22). Seedlings were grown in open containers on moist vermiculite for 5 days in complete darkness at 27°C. All manipulations of tissue and extracts were performed at 0-4°C in dim green light unless otherwise noted. Red light pulses were provided by a slide projector equipped with a red interference filter (λmax = 660 nm, 10 mm half-bandwidth) and established a Pfr (%Pfr/[Pfr] + [Pr]) × 100) of 86% (23) except where indicated. Far-red light pulses (J = 210 watts/m2) were obtained by filtering incandescent light through a cut-off filter (λ > 720 nm). Continuous far-red light irradiations were provided with fluorescent lamps (Sylvania T20T/25W/5) wrapped with one layer of red-light-transmitting cellophane (λmax = 570 nm, f = 12 watts/m2) and produced 4% Pfr at photoequilibrium.

Antibody Preparation—Polyclonal antibodies against highly purified oat phytochrome (λmax/λαv ratios > 1.0) or oat ubiquitin were raised in rabbits and purified by affinity chromatography with a column containing the corresponding protein immobilized on Affigel 10 (16). Oat ubiquitin was cross-linked to bovine γ-globulin with glutaraldehyde and boiled in the presence of 1% NaDodSO4 before injection (16, 24). Preimmune antibodies were purified by protein A affinity chromatography.

Immunoassay for Ub-P—The apical 2-3 cm of seedlings were harvested, immediately frozen in liquid nitrogen, and homogenized (1 ml/g fresh weight) in 45% ethylene glycol, 90 mM Tris-HCl, 18 mM sodium metabsulfite, 100 μM hemin, and 4 mM phenylmethylsulfonfyl fluoride, pH 8.2 (4°C) (25). Homogenates were clarified twice by centrifugation for 10 min at 50,000 × g and boiled for 2-3 min in the presence of 1% NaDodSO4. NaDodSO4 was sequestered with 6 volumes of cold 1% Triton X-100, 50 mM NaCl, 1% NaN3, 25 mM Tris-HCl, pH 7.5 (25°C). Ub-P was immunoprecipitated from the NaDodSO4-denatured homogenates (100 μl) by a 1-h incubation with 25 μl (0.25 mg/ml) of either anti-oat ubiquitin or preimmune antibodies followed by a 0.5-h incubation with 75 μl of goat anti-rabbit immunoglobulin G-coupled microspheres (obtained from Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Microspheres were collected by centrifugation for 3 min at 14,000 × g and washed three times with 500 μl of 25 mM Tris-HCl, 150 mM NaCl, 5 mM NaEDTA, pH 7.5 (25°C). The final pellets were boiled for 2-3 min in 165 μl of 1% NaDodSO4, 50 mM Tris-HCl, pH 7.5 (25°C) to release immunoprecipitated protein, and the microspheres were removed by centrifugation. The supernatants were subjected to NaDodSO4-PAGE in 8% acrylamide gels (26). Protein was transferred onto nitrocellulose for 3 h at 400 V, and the nitrocellulose was autoclaved for 30 min in transfer buffer (27). Ub-P were visualized by immunoblotting with anti-phycocyanin antibodies and conjugated horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G using nitroblue tetrazolium and 4-bromo-5-chloro-3-indolyl phosphate as substrates (11) or 125I-labeled protein A and autoradiography (16). For quantitative analyses, regions of the blot in the molecular mass range of Ub-P (>124 kDa) were scanned with a reflective densitometer (Hoeffer, San Francisco, CA). For each time point, the densitometric signal obtained with the preimmune antibody immunoprecipitate was subtracted from the signal obtained with the anti-ubiquitin antibody immunoprecipitate. This corrected signal was then compared to the immunoblot signal in the 124-kDa region for a dilution series of an oat phytochrome standard to determine the amount of conjugated phytochrome present. Ubiquitin-lysylase and heterogeneous 125I-ubiquitin-protein conjugates used to test the immunoprecipitations were prepared in wheat germ and oat extracts, respectively, as previously described (9, 28).

Phytochrome isolation—Following various irradiations, seedlings were frozen and homogenized (2 ml/g fresh weight) in 37.5% ethylene glycol, 75 mM Tris-HCl, 105 mM (NH4)2SO4, 20 mM sodium metabsulfite, and 4 mM phenylmethylsulfonfyl fluoride, pH 8.1 (4°C) (25). The extract was made 0.1% (v/v) polyethyleneimine and clarified by centrifugation for 15 min at 400,000 × g. Phytochrome was isolated from this extract by (NH4)2SO4 precipitation and hydroxypatite chromatography followed by immunoprecipitation with anti-oat phytochrome antibodies and Staphylococcus aureus cells as previously described (16). Immunoprecipitates were subjected to NaDodSO4-PAGE and Ub-P visualized by immunoblot analysis with anti-ubiquitin antibodies as previously described (16). The amount of Ub-P was measured for each sample by comparing the signal obtained with the preimmune antibody immunoprecipitate to the signal obtained with the anti-ubiquitin antibody immunoprecipitate for various dilutions of an oat phytochrome standard and determining the pool size of Ub-P during phytochrome degradation. We also showed that the commitment to form Ub-P occurs within seconds after Pfr formation and may involve aggregation of the photoreceptor.
proteins were first denatured with boiling NaDodSO₄ and purified ubiquitin-'²⁵₁-lysozyme conjugates added to crude oat presence of hemin to block possible noprecipitations. To examine the efficiency of such immunoassays, the recoveries of three different types of conjugates by isopeptidases present in the extracts (9). Because anti-ubiquitin antibodies prepared against NaDodSO₄-denatured ubiquitin effectively recognized only conjugates denatured in a similar manner (24; and data not shown), proteins were first denatured with boiling NaDodSO₄ and NaDodSO₄ sequestered with Triton X-100 before the immunoprecipitations. To examine the efficiency of such immunoprecipitations, the recoveries of three different types of ubiquitin-protein conjugates were tested: (i) ubiquitin-protein conjugates endogenous to oat extracts, (ii) heterogeneous oat '²⁶₁-ubiquitin-protein conjugates synthesized in vitro, and (iii) purified ubiquitin-'²⁸₁-lysozyme conjugates added to crude oat extracts. In all three cases, sufficient amounts of anti-ubiquitin antibodies could be added to immunoprecipitate >90% of these species (Fig. 1).

The immunoassay was applied to either unirradiated seedlings or seedlings shown previously to contain maximal levels of Ub-P (i.e. seedlings irradiated with saturating red light for 5 min, converting 86% of Pr to Pfr, and incubated in darkness for 90 min (16)). As observed previously with these seedlings (16), a red light pulse initiated the breakdown of phytochrome with 58% of the chromoprotein degraded in 90 min (Table I).

Immunoblots of the anti-ubiquitin immunoprecipitates with anti-phytochrome antibodies revealed that irradiated seedlings contained a heterogeneous array of Ub-P, observed as a series of immunoreactive species above 124 kDa (Fig. 2). In contrast, no detectable levels of Ub-P were observed in unirradiated seedlings. Similar results were observed when anti-phytochrome antibodies were used for the immunoprecipitations and anti-ubiquitin antibodies were used for the immunoblots (data not shown and Ref. 16).

The amount of phytochrome in the Ub-P fraction was determined by reflective densitometry of immunoblots similar to those in Fig. 2. Quantitation was accomplished by comparing the immunoblot signal above 124 kDa generated by the immunoprecipitates with those generated by phytochrome standards at 124 kDa. Approximately 1-2% of unmodified phytochrome routinely precipitated nonspecifically with the microspheres, being present in both immune and preimmune samples (Fig. 2). To avoid this nonspecific signal in the immunoblots, only the immunoreactive signals in the regions of the blots above 124 kDa were integrated. In unirradiated seedlings, the level of Ub-P was below the detection limit of 0.3% of the initial phytochrome pool (Table I). However, 90 min after a red light pulse, the pool size of Ub-P was 4.5% of initial phytochrome and 11% of phytochrome (both Pr and Pfr) remaining at 90 min (Table I). When relating this value to the amount of phytochrome specifically degraded in these seedlings, i.e. Pfr, ~18% of Pfr was present as ubiquitinated species after 90 min.

Relationship of Ub-P to Initial %Pfr—As a result of phytochrome’s unique spectral properties, it is possible to manipulate phytochrome degradation by altering the irradiation conditions of the seedlings (for examples, see Refs. 13, 15, 22, 32). If Ub-P are intermediates of phytochrome degradation, such changes should predictably affect the concentration of Ub-P. For example, using etiolated oat seedlings in which degradation appears specific to Pfr, reducing the amount of

### RESULTS

**Immunoprecipitation for Ub-P**—To further define kinetically the relationship of Ub-P with phytochrome degradation, an estimate of the pool size of Ub-P was needed. Previous estimates suggested that Ub-P accumulate to only a few percent of the total phytochrome pool during Pfr degradation (16). To determine this pool size more accurately, an immunoassay was developed that involved the quantitative immunoprecipitation of ubiquitin-protein conjugates from crude oat extracts using anti-ubiquitin antibodies in conjunction with goat anti-rabbit immunoglobulin G-coupled microspheres. This was followed by an immunoblot assay of Ub-P using anti-phytochrome antibodies. Crude extracts were first prepared in the presence of hemin to block possible in vitro disassembly of conjugates by isopeptidases present in the extracts (9). Because anti-ubiquitin antibodies prepared against NaDodSO₄-denatured ubiquitin effectively recognized only conjugates denatured in a similar manner (24; and data not shown), proteins were first denatured with boiling NaDodSO₄ and NaDodSO₄ sequestered with Triton X-100 before the immunoprecipitations. To examine the efficiency of such immunoprecipitations, the recoveries of three different types of ubiquitin-protein conjugates were tested: (i) ubiquitin-protein conjugates endogenous to oat extracts, (ii) heterogeneous oat '²⁶₁-ubiquitin-protein conjugates synthesized in vitro, and (iii) purified ubiquitin-'²⁸₁-lysozyme conjugates added to crude oat extracts. In all three cases, sufficient amounts of anti-ubiquitin antibodies could be added to immunoprecipitate >90% of these species (Fig. 1).

The immunoassay was applied to either unirradiated seedlings or seedlings shown previously to contain maximal levels of Ub-P (i.e. seedlings irradiated with saturating red light for 5 min, converting 86% of Pr to Pfr, and incubated in darkness for 90 min (16)). As observed previously with these seedlings (16), a red light pulse initiated the breakdown of phytochrome with 58% of the chromoprotein degraded in 90 min (Table I). Immunoblots of the anti-ubiquitin immunoprecipitates with anti-phytochrome antibodies revealed that irradiated seedlings contained a heterogeneous array of Ub-P, observed as a series of immunoreactive species above 124 kDa (Fig. 2). In contrast, no detectable levels of Ub-P were observed in unirradiated seedlings. Similar results were observed when anti-phytochrome antibodies were used for the immunoprecipitations and anti-ubiquitin antibodies were used for the immunoblots (data not shown and Ref. 16).

The amount of phytochrome in the Ub-P fraction was determined by reflective densitometry of immunoblots similar to those in Fig. 2. Quantitation was accomplished by comparing the immunoblot signal above 124 kDa generated by the immunoprecipitates with those generated by phytochrome standards at 124 kDa. Approximately 1-2% of unmodified phytochrome routinely precipitated nonspecifically with the microspheres, being present in both immune and preimmune samples (Fig. 2). To avoid this nonspecific signal in the immunoblots, only the immunoreactive signals in the regions of the blots above 124 kDa were integrated. In unirradiated seedlings, the level of Ub-P was below the detection limit of 0.3% of the initial phytochrome pool (Table I). However, 90 min after a red light pulse, the pool size of Ub-P was 4.5% of initial phytochrome and 11% of phytochrome (both Pr and Pfr) remaining at 90 min (Table I). When relating this value to the amount of phytochrome specifically degraded in these seedlings, i.e. Pfr, ~18% of Pfr was present as ubiquitinated species after 90 min.

**Relationship of Ub-P to Initial %Pfr**—As a result of phytochrome’s unique spectral properties, it is possible to manipulate phytochrome degradation by altering the irradiation conditions of the seedlings (for examples, see Refs. 13, 15, 22, 32). If Ub-P are intermediates of phytochrome degradation, such changes should predictably affect the concentration of Ub-P. For example, using etiolated oat seedlings in which degradation appears specific to Pfr, reducing the amount of

### Table I

| Pool size estimate of ubiquitin-phytochrome conjugates during Pfr degradation |
|------------------------------|-----------------|-----------------|
| Tissue | µg/ml extract | % |
| Initial | 7.3 ± 1.5 | 42.4 ± 5.1 |
| After 90 min | 0.80 ± 0.25 | (11.1 ± 1.0) |
| Ubiquitin-phytochrome conjugates | | |
| Initial | 0.80 ± 0.25 | (4.5 ± 0.5) |
| After 90 min | 0.80 ± 0.25 | (18.4 ± 2.4) |

**5000 Ubiquitin-Phytochrome Conjugates**

**Fig. 1. Immunoprecipitation of ubiquitin-protein conjugates with anti-ubiquitin antibodies.** Crude extracts were prepared from etiolated oat seedlings without (C, D) or with the addition of ubiquitin-'²⁶₁-lysozyme conjugates (G, H) or heterogeneous '²⁸₁-ubiquitin-protein conjugates (D, A) as described under Experimental Procedures. Protein in the extracts was denatured with NaDodSO₄, and NaDodSO₄ sequestered with Triton X-100. Ubiquitin conjugates were immunoprecipitated with various amounts of either rabbit anti-oat ubiquitin antibodies (open symbols) or preimmune antibodies (closed symbols) in conjunction with goat anti-rabbit antibody coated microspheres. Recovery of total ubiquitin-protein conjugates was quantitated by immunoblotting with anti-ubiquitin antibodies while the recovery of ubiquitin-'²⁶₁-lysozyme conjugates and heterogeneous '²⁸₁-ubiquitin-protein conjugates was quantitated by scintillation counting.
Pfr degraded by reducing the amount of Pfr initially produced should alter the accumulation of Ub-P. When seedlings were irradiated for 1 min with various fluence rates of red light producing a range of initial %Pfr from 0 to 86%, the amount of phytochrome degraded after 90 min was concomitantly affected (Fig. 3). The final amount of phytochrome degraded was close to the amount of Pfr initially produced in each case. Analysis of Ub-P present in vivo 90 min after the red light pulses demonstrated that conjugate levels were dependent on the initial %Pfr (Fig. 3). Below an initial %Pfr of 10%, conjugates were just detectable, and their abundance increased substantially as the initial %Pfr increased to 86%.

Phytochrome Degradation under Continuous Far-red Light—In oat, rapid phytochrome degradation occurs with zero order kinetics with the rate of degradation becoming independent of Pfr content when the level of Pfr is maintained above a threshold (15). The level of Pfr can be kept above this threshold (~1% of initial Pfr) with a continuous far-red irradiation that maintains approximately 4% of phytochrome in the Pfr form (Fig. 4A). The kinetic of protein breakdown was equivalent to that following a saturating red light pulse (16); exhibiting zero order kinetics, with 50% of phytochrome lost within 100 min (as determined by spectrophotometry and immunoblotting). This irradiation scheme also induced the accumulation of Ub-P in vivo (Fig. 4B, left panel). A series of conjugates, ranging in size from 129 to 170 kDa, was detected after 5 min, reached a maximum after 90 min and declined thereafter (see Fig. 5). From their apparent molecular masses, we estimated that the predominant species contained from one to seven ubiquitin moieties per phytochrome monomer. The distribution and amount of Ub-P appeared similar to that induced by a red light pulse (16). In fact, direct comparison of seedlings from the same container which were either irradiated with a red light pulse and incubated in the dark for 90 min or exposed for 90 min to continuous far-red light demonstrated that the accumulation of conjugates under the two light regimes was similar (Fig. 4B, right panel). In this case, Ub-P accumulation was not dependent on the levels of Pfr as long as rapid phytochrome degradation was maintained.

Loss of Ub-P—Schafer et al. (15) previously showed that phytochrome degradation induced by continuous far-red light can be arrested by discontinuing the irradiation. Similarly, we found that when oat seedlings irradiated for 90 min with continuous far-red light were transferred into darkness, subsequent phytochrome degradation ceased (Fig. 4A). Analysis of Ub-P indicated that they were rapidly lost in vivo after the transfer to darkness (Fig. 5). Each molecular mass species disappeared with the same rate suggesting that the loss of conjugates was not affected substantially by the number of ubiquitins attached. The rate of Ub-P disappearance was first order with a half-life of 30 min (Fig. 5). In contrast, maintaining the plants under continuous far-red light for an additional 90 min allowed for continued phytochrome breakdown and, concomitantly, maintained a high level of conjugates (Fig. 5). Thus, phytochrome degradation and Ub-P accumulation both required the continued production of Pfr by far-red light.

Formation of Ub-P during Cycled Pr Degradation—In certain seed lots of oat, degradation of “cycled Pr” (i.e. Pr produced by the phototransformation of Pfr back to Pr) has been observed (22, 31, 32). Degradation of cycled Pr follows
investigated. As with other seed lots, these oat seedlings the degradation of cycled Pr and accumulation of Ub-P was degradation is unknown.

for 90 min and then transferred to darkness (arrow) (●). At various times, tissue was rapidly frozen and homogenized, and the phytochrome content in the crude extract was determined spectrophotometrically (G, ●, ■) or by immunoblot analysis (gel lanes). Immunoblot analysis was performed with anti-oat phytochrome antibodies after subjecting equal volumes of crude extracts to Na-DodSO₄-PAGE. Only the region of the blot surrounding the 124-kDa phytochrome monomer is shown. 

A kinetics of phytochrome degradation and accumulation of Ub-P in etiolated oat seedlings during continuous far-red light irradiation. A, kinetics of phytochrome degradation. Etiolated seedlings were either kept in darkness (■), irradiated continuously with far-red light (○, gel lanes), or irradiated with far-red light for 90 min and then transferred to darkness (arrow) (●). At various times, tissue was rapidly frozen and homogenized, and the phytochrome content in the crude extract was determined spectrophotometrically (G, ●, ■) or by immunoblot analysis (gel lanes). Immunoblot analysis was performed with anti-oat phytochrome antibodies after subjecting equal volumes of crude extracts to Na-DodSO₄-PAGE. Only the region of the blot surrounding the 124-kDa phytochrome monomer is shown. B, analysis of Ub-P accumulation. Left panel, at the times indicated after the onset of continuous far-red irradiation (FR), seedlings were frozen and homogenized, and Ub-P were isolated and visualized by immunoblot analysis with anti-ubiquitin antibodies as described in Fig. 3. Right panel, in a separate experiment, seedlings from the same container were either irradiated with a red light pulse and incubated in the dark for 5 min (R) or irradiated with continuous far-red light for 90 min (FR). Seedlings were then frozen, and Ub-P were visualized as described above. The amount of immunoprecipitated phytochrome applied to each gel lane in both panels represents the relative phytochrome content in the corresponding crude extract (see panel A). Arrowheads indicate the position of the 124-kDa phytochrome monomer.

zero order kinetics and can result in the loss of up to 35% of the chromoprotein. Both kinetic and inhibitor studies indicate that cycled Pr is rapidly degraded by the same mechanism as Pfr (32). The cause of the seed lot specificity of cycled Pr degradation is unknown.

Using seedlings that display cycled Pr degradation, both the degradation of cycled Pr and accumulation of Ub-P was investigated. As with other seed lots, these oat seedlings rapidly degraded Pfr and synthesized Ub-P after a 3-s red light flash that converted 86% of Pr to Pfr (Fig. 6, right panels). After a 60-min dark incubation, 30% of Pfr was degraded with the remaining Pfr degraded within 4 h. These seedlings also degraded cycled Pr if Pfr was photoconverted back to Pr 5 min after the initial red light flash (Fig. 6). Similar to previous studies (31, 32), 28% of phytochrome was degraded within 60 min. Such irradiations also induce the synthesis of Ub-P (Fig. 6). In fact, the molecular mass distribution of Ub-P produced during cycled Pr degradation was indistinguishable with that elicited during Pfr degradation although the steady state levels were lower (Fig. 6). In con-
Contrast, no degradation of phytochrome nor formation of Ub-P occurred after irradiation with a pulse of far-red light alone.

Cycled Pr degradation can be inhibited by shortening the time between the red and far-red flashes (33) and/or by lowering the temperature of the tissue during the photocycling (Fig. 6). In the case where complete photocycling was accomplished within 18 s, the amount of phytochrome ultimately degraded was reduced to 19%. Likewise, we observed that the level of Ub-P was concomitantly lowered (Fig. 6, right panels). Temperature effects on cycled Pr degradation were investigated by reducing the temperature of the seedlings to 4 °C during the irradiations and subsequently raising the temperature back to 23 °C during the following 60-min dark incubation (Fig. 6, left panels). Seedlings irradiated at 4 °C with red light alone degraded Pfr and synthesized Ub-P similar to unchilled seedlings indicating that the cold treatment did not adversely affect the tissue. However, when seedlings containing cycled Pr were examined, chilling during the light treatments affected both phytochrome degradation and the accumulation of Ub-P. Tissue irradiated sequentially with red and far-red light with no dark interval did not degrade phytochrome nor synthesize Ub-P. Seedlings sequentially irradiated with red and far-red light separated by a 5-min dark interval degraded less phytochrome and accumulated less Ub-P than red-irradiated seedlings.

Association of Ub-P with Pelletable Fractions—Within seconds after irradiating etiolated oat seedlings with red light, phytochrome changes rapidly (Δt ~ 2 s) from a disperse distribution in the cytoplasm to one where phytochrome is sequestered (or aggregated) into numerous discrete areas (34–37). Phytochrome can be extracted in a similarly aggregated form, termed pelletable, if red light-irradiated tissue is homogenized in buffers containing millimolar concentrations of divalent cations (15, 29, 37–39). Such pelletable phytochrome has the new property of sedimenting at relatively low g forces. Because a majority of sequestered (or pelletable) phytochrome disappears during Pfr degradation, it has been proposed that phytochrome aggregation may be one of the early steps during the molecule’s breakdown (15, 34, 39).

To examine the possibility that Ub-P are preferentially associated with aggregated phytochrome, both the pelletable and soluble fractions of phytochrome were isolated after a red irradiation and assayed for phytochrome content and the presence of Ub-P. As observed previously (29, 38), <10% of phytochrome in unirradiated oat seedlings was pelletable when extracted in 10 mM Mg²⁺ (Fig. 7A) with the majority of phytochrome still soluble (>90%). Following a 5-min red light pulse, 60% of total phytochrome became pelletable. As Pfr degradation proceeded, loss of Pfr occurred initially and to

---

**FIG. 6. Appearance of Ub-P during cycled Pr degradation.** Oat seedlings that exhibit cycled Pr degradation were irradiated at 4 or 23°C with red (R) and/or far-red (FR) light as indicated and incubated for 60 min at 27°C in darkness. The seedlings were then homogenized and Ub-P were isolated and visualized by immunoblot analysis with anti-ubiquitin antibodies as in Fig. 3. **Upper panels,** the amount of phytochrome degraded 60 min after the various irradiations; **lower panels,** the relative amount of Ub-P that accumulated 60 min after the onset of the various irradiations. D = darkness.

**FIG. 7. Association of Ub-P with the pelletable and soluble fractions of phytochrome during Pfr degradation.** Etiolated oat seedlings were irradiated for 3 min with red light and then incubated in darkness. At various times, seedlings were homogenized without prior freezing in a Mg²⁺-containing buffer and the pelletable and soluble fractions of phytochrome separated by centrifugation. A, degradation kinetics of pelletable and soluble phytochrome. Phytochrome content was determined spectrophotometrically in the crude pelletable (Δ) and soluble (□) fractions and the values added together to obtain total phytochrome content (○). **Closed symbols** indicate phytochrome content in each fraction immediately before the red light irradiation or for unirradiated tissue kept in darkness. B, kinetics of Ub-P accumulation in the pelletable and soluble phytochrome fractions. Immediately before or following various times after Pfr formation, Ub-P were isolated from the soluble and pelletable pools of phytochrome and visualized by immunoblot analysis with anti-ubiquitin antibodies as described in Fig. 3. The amount of phytochrome applied to each gel lane represented the relative amount phytochrome content in the corresponding pelletable or soluble fraction (see panel A). Arrowheads indicate the position of the 124-kDa phytochrome monomer. P and S = pelletable and soluble phytochrome, respectively; UN = samples obtained from tissue immediately before red light irradiation.
the greatest extent from the pelletable fraction. In fact, phytochrome breakdown for the first 90 min could be accounted for almost entirely by loss from the pelletable fraction. The content of Pfr in the soluble fraction remained apparently constant over most of the time course of Pfr breakdown with losses observed only after most of the pelletable phytochrome was degraded (Fig. 7A).

Analysis of Ub-P in the pelletable and soluble phytochrome fractions revealed that most conjugates were associated with the pelletable fraction (Fig. 7B). They could be detected in the pelletable fraction within 5 min after the onset of the red irradiation with their appearance and disappearance coinciding with the loss of pelletable phytochrome. Initially, few Ub-P were detected in the soluble fraction (from 0 to 30 min), but they accumulated to significant levels at later times coinciding with loss of phytochrome from that fraction (Fig. 7A).

**DISCUSSION**

Based on the appearance of Ub-P during red light-induced phytochrome degradation and ubiquitin's established role in protein catabolism (5, 6), we had proposed that phytochrome is degraded in vivo by a ubiquitin-dependent proteolytic pathway (16). The data reported here support this hypothesis by including observations that: 1) When irradiating seedlings with either red light pulses or continuous far-red light, the accumulation of Ub-P correlates with the amount of Pfr degraded; 2) Ub-P are continually synthesized during phytochrome degradation and disappear when phytochrome degradation ceases; 3) in seedlings that display cycled Pr degradation, the levels of Ub-P synthesized correlate with the amount of cycled Pr degraded; and 4) Ub-P are associated first and to the greatest extent with the form of phytochrome (aggregated) that is most rapidly degraded. While additional interpretations are possible, these data are consistent with the possibility that phytochrome is rapidly degraded via ubiquitinated intermediates. If so, phytochrome would be the first intracellular protein identified that is degraded by the ubiquitin-proteolytic pathway in vivo. These data also would provide evidence for a physiological role of ubiquitin conjugation in plants. The ATP requirement for both ubiquitin ligation (7) and degradation of ubiquitin-protein conjugates (7) would explain the energy dependence reported for phytochrome breakdown (40).

The pool dynamics of Ub-P provide one of the first descriptions of how a specific ubiquitin conjugate accumulates and disappears in vivo. As a result, several observations deserve notice. First, the pool size of Ub-P indicates that substantial amounts of conjugated phytochrome (~5% of the initial phytochrome pool or 11% of the pool remaining 90 min after the red irradiation) accumulates in vivo after Pfr formation. (We note that this estimate for Ub-P may be conservative given that conjugation of ubiquitin to phytochrome diminishes the immunorecognition of Ub-P by anti-phytochrome antibodies (data not shown), and the likelihood that Ub-P transfer less efficiently than phytochrome during western blotting as a result of its bifurcated structure and larger size.) By comparison, the most abundant ubiquitinated protein reported to date, hH2A, comprises up to 11% of the histone H2A pool (41). Turnover studies of hH2A indicate that ubiquitination is not involved in the catabolism of this histone (42). By switching the use of the antibodies employed in the immunoblot assay, we estimated that at maximum between 1 and 5% of total ubiquitin becomes conjugated to phytochrome during Pfr degradation (data not shown). Thus, only a small percentage of the ubiquitin pool may be involved in degrading phytochrome at any one time.

Second, during both the synthesis and loss of Ub-P, all size classes of Ub-P appear and disappear simultaneously (Figs. 4 and 5). If ubiquitins were added in a processive manner, one would expect a progressive increase over time in the molecular mass distribution of Ub-P (Figs. 4 and 7). Likewise, if disappearance was preferential for multiply ubiquitinated species, one would expect a preferential loss of the larger size classes of Ub-P (Figs. 4 and 5). Because accumulation and disappearance of Ub-P appears to be independent of the number of ubiquitins attached, it is possible that one or more ubiquitins are added either rapidly or in one step to individual chromoproteins and that loss is independent of the number of ubiquitins attached.

Third, the phenomena of cycled Pr degradation and ubiquitination indicate that a portion of phytochrome changes either in location or in its physicochemical characteristics after photoconversion to Pfr resulting in molecules committed to degradation/ubiquitination in vivo regardless of the spectral form. Kinetics of cycled Pr conjugation/degradation at 25 and 4 °C indicate that the commitment is rapid, occurring within the 18 sec irradiation scheme used to generate cycled Pr (Fig. 6). We predict that the commitment reaction is likely to be even more rapid because only 3 s of far-red light was required to convert >95% of Pfr back to Pr. This commitment could result solely from photocycling the chromoprotein to Pfr and back to Pr. However, the observations that seedlings photocycled at low temperatures neither degrade phytochrome nor synthesize Ub-P would discount this possibility (Fig. 6). Alternatively, the commitment may involve an interaction of Pfr with other components of the cell that is not quickly reversed by a subsequent conversion back to Pr. The temperature dependence of cycled Pr degradation/conjugation would suggest that this interaction is in part enzymatic. One rapid reaction known to occur in vivo within the time frame required for committing cycled Pr to both degradation and ubiquitination is phytochrome aggregation (29, 35, 38). Phytochrome aggregation is strongly temperature-dependent, requires energy, is rapidly induced (t½ = 2 s at 25 °C) after Pfr formation, and is slowly reversed after photoconversion back to Pr (t½ = 25 min at 25 °C) (29, 31, 35, 38). Both the temperature dependence and energy requirement indicate that metabolic reaction(s) subsequent to phototransformation are involved. Thus, conjugation and degradation of cycled Pr may be a consequence of the chromoprotein's aggregation. The preferential association of Ub-P with pelletable phytochrome and the observations that cycled Pr aggregates like Pr (31, 36-38) supports this possibility.

The connection of phytochrome aggregation and ubiquitin conjugation to phytochrome breakdown further supports the possibility that aggregation is involved in the photoreceptor's degradation (31) and provides a possible mechanism for such degradation. Immunocytochemical data indicate that these aggregated regions are cytoplasmic and consist of ~1 μm amorphous granules lacking a delineated membrane (36, 37). Similar structures termed inclusion bodies have been observed in both prokaryotic and eukaryotic cells which contain large amounts of abnormal proteins (43, 44). These inclusion bodies are formed by the aggregation of abnormal proteins and appear to be the sites where such proteins are catabolized. Based on the similarities of aggregation of a "normal" protein like phytochrome with abnormal proteins, it is possible that such aggregates are the sites where all cytoplasmic proteins are degraded. However, we have been unable to detect other ubiquitinated proteins that are pelletable (see above) indicat-
ing that this is not a general phenomenon but rather specific for phytochrome (data not shown). The processes that underly aggregation are unknown. Unlike phytochrome, aggregation of abnormal proteins appears to result from spontaneous precipitation rather than active processes (43). Our studies show that Ub-P also appear in soluble phytochrome pool but at much later times than in the pelletable pool (Fig. 7). This could result from conjugation of ubiquitin to soluble chromoproteins. Alternatively, because phytochrome aggregates gradually become smaller during Pfr degradation and finally disappear (34), it is possible that soluble Ub-P are actually aggregated species now too small to pellet.

Asuming that all Pfr is degraded via ubiquitinated intermediates, a minimal model for Pfr-specific phytochrome degradation can be proposed:

\[
Pr \rightarrow Pfr \rightarrow Ub-P \rightarrow \text{amino acids} \rightarrow Ub
\]

Based on a maximal pool size of Ub-P at 5% of initial phytochrome and previous data showing that the overall rate of Pfr degradation is zero order with 50% of Pfr lost in 80 min (Table I; 16), the half-life of Ub-P after a red light pulse would be ~5 min if loss is first order (calculations not shown). An indirect attempt to measure it by irradiating seedlings with continuous far-red light followed by darkness gave a 30-min half-life for Ub-P (Fig. 5), much longer than the estimate. This reason for the difference is unknown although several possibilities exist. These include the possibility that (i) only a portion of phytochrome is degraded via ubiquitinated intermediates. For example, using a 30-min half-life for Ub-P, we calculate that only 17% of Pfr would be degraded by the ubiquitin pathway. (ii) Turnover of Ub-P following a prolonged far-red irradiation may be different than following a red light pulse although the rates of phytochrome breakdown induced by the irradiations are the same. This could result from the fact that following a far-red irradiation, 96% of phytochrome would be in the cycled Pfr form which can assume a different intracellular location than Pfr (15, 29). (iii) And finally, it is possible that most loss of Ub-P after switching from far-red light to darkness does not represent degradation but rather disassembly of Ub-P. This would account for the reproducible observation that spectrophotometrically detectable phytochrome was not reduced after discontinuing the far-red irradiation (Fig. 4A). The enzymes responsible for disassembly, ubiquitin-protein lysases, have been detected in oat seedling extract (9) and are able to disassemble partially purified oat Ub-P in vitro (data not shown). Because most Ub-P produce under continuous far-red light irradiation may be Pr conjugates, it is possible that these proteins are recognized as incorrectly conjugated by ubiquitin-protein lysases and returned to their unmodified form.

From their observations of cycled Pr degradation, Stone and Pratt (32) proposed that rapid phytochrome degradation is not dependent on the spectral form of the photoreceptor, but a consequence of its intracellular location, aggregated or soluble. Consistent with their hypothesis, a pathway of rapid phytochrome degradation in agreement with the experimental data reported here can be proposed. Following photoconversion of Pr to Pfr, the photoreceptor is modified inducing its rapid aggregation within the cytoplasm. As a result of this aggregation, the chromoprotein is recognized by the ubiquitin conjugating with one or more ubiquitins, and degraded by proteases specific for ubiquitin-protein conjugates. Thus, formation of Ub-P may result directly from the intracellular location of the chromoprotein and not solely from the physicochemical difference between the Pr and Pfr forms. In the case of cycled Pr degradation, two competing reactions may exist, one of disaggregation and the other of ubiquitin conjugation to the aggregated species. The relative rates of the two could determine how much cycled Pr is conjugated with ubiquitin and ultimately degraded. The process(es) underlying Pfr aggregation are unknown but they may be central steps in committing phytochrome to rapid degradation.

REFERENCES

1. Goldberg, A. L., and St. John, A. C. (1976) Annu. Rev. Biochem. 45, 747-803
2. Hershko, A., Heller, H., Kytan, E., Kukli, G., and Rose, I. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7021-7025
3. Bachman, A., Finley, D., and Varshavsky, A. (1986) Science 234, 172-188
4. Beppe, S., Wels, H., and Rechsteiner, M. (1986) Science 234, 584-588
5. Finley, D., and Varshavsky, A. (1985) Trends Biochem. Sci. 10, 342-347
6. Rieger, G., and Hoch, A. (1988) J. Biochem. 268, 11357-11360
7. Hough, R., and Rechsteiner, M. (1986) J. Biol. Chem. 261, 2391-2399
8. Matsui, S.-E., Sandberg, A. A., Nag, S., Sonn, B. K., and Goldstein, G. (1983) Proc. Natl. Acad. Sci. U. S. A. 79, 1338-1339
9. Vierstra, R. D., and Sullivan, M. L. (1988) Biochemistry 27, 3329-3332
10. Hans, A. L., and Bright, P. M. (1985) J. Biol. Chem. 260, 12464-12473
11. Vierstra, R. D., Langen, S. M., and Hans, A. L. (1986) J. Biol. Chem. 261, 12015-12020
12. Horwich, J. R. E., and Kronenberg, G. H. M. (eds) (1986) Photomorphogenesis in Plants Martinus Nijoff Publishing, Dordrecht, The Netherlands
13. Quail, P. H., Schaffer, E., and Marre, D. (1973) Plant Physiol. 52, 129-131
14. Pratt, L. H., Kidd, G. H., and Colemar, R. A. (1974) Biochim. Biophys. Acta 369, 93-107
15. Schare, E., Lassig, T. U., and Schaper, P. (1975) Photosynth. Photochem. 22, 193-202
16. Jentsch, S., Marm, E., and Pratt, P. H. (1983) Plant Physiol. 72, 324-327
17. Coefi, J. T., Hershey, H. P., and Quail, P. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 80, 2244-2249
18. Levinger, L., and Varshavsky, A. (1982) Cell 28, 375-385
19. Siegemann, M., Bond, M. W., Kiesel, W. M., St. John, T., South, H. T., and Rechsteiner, M. (1986) Science 231, 825-830
20. Jentsch, S., McGrath, J. P., and Rechsteiner, A. (1987) Nature 329, 131-134
21. Goebel, M., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. B. (1988) Science 241, 1331-1335
22. Dooskin, R. H., and Mancinelli, A. L. (1988) Bull. Torrey Bot. Club 95, 474-487
23. Vierstra, R. D., and Quail, P. H. (1983) Plant Physiol. 72, 264-267
24. Hershko, A., Kytan, E., Cerro, A., and Sato, S. (1982) J. Biol. Chem. 257, 13896-13970
25. Vierstra, R. D., and Quail, P. H. (1983) Biochemistry 22, 2498-2505
26. Lasemn, U. K. (1970) Nature 227, 680-685
27. Swedower, P. S., Finley, D., and Varshavsky, A. (1986) Anub. Biochem. 166, 147-153
28. Vierstra, R. D. (1987) Plant Physiol. 84, 332-336
29. Pratt, L. H., and Marre, D. (1976) Plant Physiol. 58, 686-692
30. Little, J. C., Kelley, M. J., and Lagarias, J. C. (1983) J. Biol. Chem. 258, 11025-11031
31. Mackenzie, J. M., Briggs, W. R., and Pratt, L. H. (1978) Planta 141, 129-134
32. Stone, H. J., and Pratt, L. H. (1979) Plant Physiol. 63, 680-682
33. Jabbene, M. (1969) Planta 149, 91-96
34. Mackenzie, J. M., Colemar, R. A., Briggs, W. R., and Pratt, L. H. (1979) Proc. Natl. Acad. Sci. U. S. A. 72, 799-803
35. Mccurry, D. W., and Pratt, L. H. (1986) Planta 167, 330-336
36. Sprem, V., Otto, V., and Schafner, E. (1982) Planta 168, 299-304
37. Mccurry, D. W., and Pratt, L. H. (1987) J. Cell Biol. 103, 2541-2550
38. Quail, P. H., and Briggs, W. R. (1978) Plant Physiol. 62, 775-778
39. Bossard, J., Marre, D., and Briggs, W. R. (1974) Plant Physiol. 54, 272-276
40. Butler, W. L., and Lane, H. C. (1965) Plant Physiol. 40, 13-17
41. West, M. H. P., and Bonner, W. M. (1980) Biochemistry 19, 3223-3228
42. Wu, R. S., Kohn, K. W., and Bonner, W. M. (1981) J. Biol. Chem. 256, 5916-5920
43. Ploemen, T. E., Marno, J. M., and Goldberg, A. L. (1975) J. Biol. Chem. 250, 1112-1122
44. Kelly, J. E., Epling, J. D., and Goldberg, A. L. (1981) J. Biol. Chem. 256, 8344-8444