Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 5778–5780, August 1989
Biochemistry

Self-assembly of synthetic phytochrome holoprotein *in vitro*

(plant photoreceptor/phytochrome biosynthesis/phycocyanobilin attachment/bilin C-S lyase)

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Communicated by Eric E. Conn, May 8, 1989 (received for review April 17, 1989)

ABSTRACT **The phytochrome holoprotein of plants requires a covalently bound linear tetrapyrrole (bilin) prosthetic group for its photoreceptor function. Here we show that synthetic phytochrome apoprotein prepared by transcription and translation of an *Avena* phytochrome cDNA construct combines *in vitro* with phycocyanobilin, an analog of the natural chromophore, to produce a photoactive holoprotein. These results indicate that holoprotein assembly is an "autocatalytic" process.**

Phytochrome is a photoreceptor that mediates a wide variety of growth and developmental responses in plants at all stages of the life cycle (1). The native holoprotein consists of two polypeptide subunits of ≈1100 amino acid residues, each with a covalently associated linear tetrapyrrole (bilin) prosthetic group (2). Light absorption by the bilin prosthetic group effects the reversible conversion between the red-absorbing Pr form and the far-red-absorbing Pfr form of phytochrome, a process that initiates photomorphogenesis in plants.

We have recently reported (3) that synthesis of spectrally active holoprotein occurs posttranslationally when apophytochrome obtained from tetrapyrrole-deficient *Avena* seedlings is incubated with phycocyanobilin, the cleaved prosthetic group of the cyanobacterial photosynthetic accessory antenna protein C-phycocyanin. Because it is possible that a bilin lyase activity copurified with the phytochrome apoprotein, we prepared synthetic phytochrome apoprotein in a nonplant system by transcription and translation of an *Avena* phytochrome cDNA construct. Incubation of phytochrome apoprotein with phycocyanobilin produced a photoreversible holoprotein. These results indicate that bilin attachment to apophytochrome requires no additional enzyme activity for the generation of photoactive holoprotein.

MATERIALS AND METHODS

Plasmids. Two *Avena* phytochrome clones, a cDNA clone pAP3.2 (4) and a genomic clone pGP8.2-2 (5), were used to construct a full-length clone. pAP3.2 lacks a small region of 5′ coding sequence that is contained in pGP8.2-2. After restriction of pGP8.2-2 with *Acc* I, 5′ overhangs were filled in with Klenow fragment, and a 576-base-pair (bp) fragment was purified from the mixture of fragments after separation in an agarose gel; the 576-bp fragment contains the ATG initiator codon and a *Kpn* I site that occurs only once within the coding sequences. This 5′ phytochrome coding sequence was inserted into a pGEM-4 transcription vector in the orientation that would yield sense transcripts from the T7 RNA polymerase promoter. The resulting construct was named pPC-2M. pPC-2M and pAP3.2 were doubly restricted with *Kpn* I and *Eco*RI and ligated without further purification. Tetracycline-sensitive, ampicillin-resistant clones were screened by restriction analysis. The construct containing the full-length coding region for *Avena* phytochrome in a sense orientation to the T7 promoter was named pPC3.

RNA Preparation. Capped RNAs were transcribed from *Pvu* I-linearized pPC3 templates using T7 RNA polymerase in the presence of a 7-methylguanosinium 5′-(guanosine 5′-triphosphate) (5′ 7meGpppG 3′) cap analog (6). Visualization of RNA on ethidium bromide-stained formaldehyde gels showed that >95% of the product was of one length of ≈3.65 kilobases (kb); the RNA was also quantitated on these gels. Poly(A)$^+$ RNA from etiolated *Avena* seedlings was prepared using the guanidinium/cesium chloride method (7) similar to that described (8).

***In Vitro* Translations.** Rabbit reticulocyte lysate was prepared, and *in vitro* translation was performed essentially as recommended (9). mRNA at 20 μg/ml was translated for 1 hr at 30°C under green safelight (3) in the presence of [^{35}S]methionine at 900 μCi/ml (3000 Ci/mmol; 1 Ci = 37 GBq), bovine liver tRNA at 100 μg/ml, wheat germ tRNA at 100 μg/ml, and RNasin at 300 units per ml (Promega).

Immunoprecipitations. Immunoprecipitations of phytochrome polypeptides from translation mixtures were performed as described (10), by using a rabbit antibody raised against *Avena* phytochrome and fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem). The immunoprecipitation protocol was evaluated by its ability to separate ^{35}S-labeled phytochrome from proteins translated *in vitro* from *Avena* poly(A)$^+$ RNA.

Assay for Photoactive Holoprotein Formation. All procedures described below were performed under green safelight. Translation mixtures containing ^{35}S-labeled phytochrome were cooled on ice, and half of the translation mixture was diluted 1:1 with 2 μM phycocyanobilin in 50 mM Tris·HCl, pH 7.8/5 mM EDTA/1.4 mM 2-mercaptoethanol, and half was diluted with buffer only as a control. After incubation for 1 hr at 5°C in the dark, half of each sample was irradiated with red light and half was irradiated with far-red light (11). Each of these samples was diluted 1:1 with freshly prepared subtilisin (5 μg/ml) in 25 mM Tris·HCl, pH 8/1 mM EDTA/50% (vol/vol) ethylene glycol, incubated for 60, 120, and 240 min at 5°C, and then quenched by adding 1/10 vol of 100 mM phenylmethylsulfonyl fluoride. Samples were held at −80°C until ready to load onto gels. After SDS/PAGE, gels were treated with EN^{3}HANCE (New England Nuclear), and fluorography was performed at −80°C by using a Dupont Cronex intensifying screen.

RESULTS AND DISCUSSION

Although a full-length cDNA was not available for expression of phytochrome *in vitro*, partial cDNA and genomic clones for *Avena* have been described (4, 5). The 5′ sequences from a genomic clone and the remainder of the coding region from a partial cDNA clone were subcloned behind the T7 RNA polymerase promoter of pGEM-4 (Fig. 1). When RNA produced from this transcription plasmid was used for translation in a reticulocyte lysate system, it produced a polypeptide of 124 kDa as shown by SDS/PAGE analysis (Fig. 2). That

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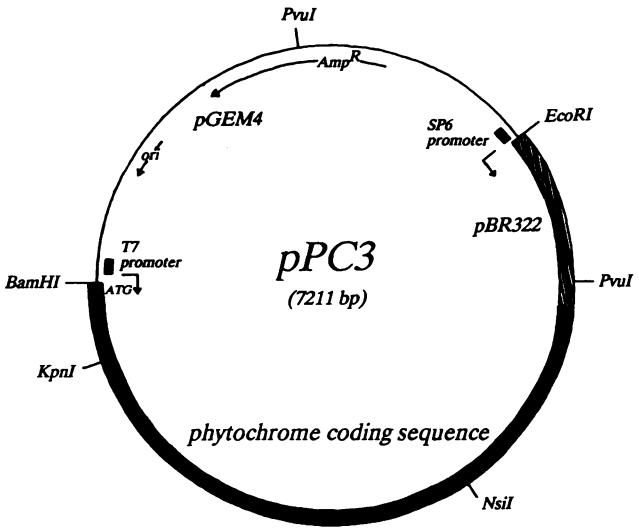

FIG. 1. Transcription plasmid pPC3 containing the complete coding region for phytochrome.

this polypeptide was phytochrome was confirmed by its immunoprecipitation with phytochrome antiserum, which was prevented by adding excess unlabeled phytochrome, and by its identical mobility with the immunoprecipitated polypeptide from *in vitro* translation of *Avena* poly(A)$^+$ RNA (Fig. 2). To obtain a majority of full-length phytochrome polypeptides, it was necessary to titrate the input RNA to a low level, to reduce translation time to 60 min, and to

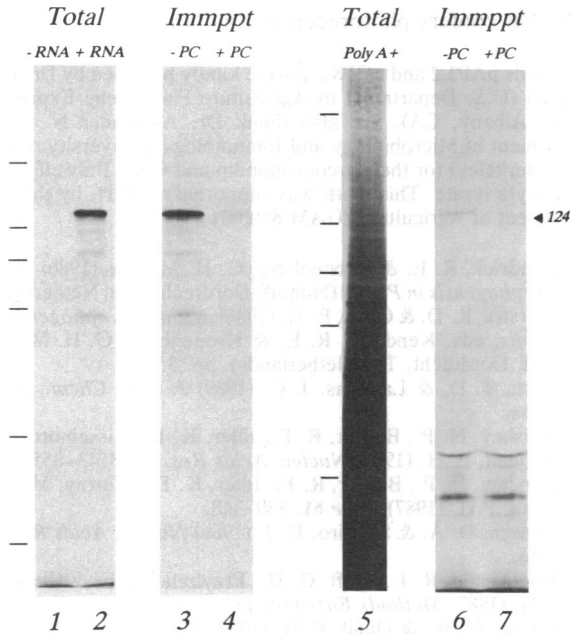

FIG. 2. *In vitro* translation of pPC3 RNA transcripts and immunochemical identification of phytochrome polypeptides. ^{35}S-labeled proteins synthesized *in vitro* in the absence of added RNA (lane 1) and in the presence of RNA synthesized by T7 polymerase from pPC3 (lane 2). Phytochrome-related polypeptides were immunoprecipitated (Immppt) from translation mixtures in the absence (lane 3) and presence (lane 4) of unlabeled purified phytochrome (PC) from etiolated *Avena sativa* seedlings. ^{35}S-labeled proteins synthesized *in vitro* from *Avena* poly(A)$^+$ RNA (lane 5) were also immunoprecipitated in the absence (lane 6) and presence (lane 7) of unlabeled competitor phytochrome. Molecular mass standards at left represent myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). The arrowhead at right indicates the apparent molecular mass (in kDa) of the phytochrome polypeptide.

preincubate all components of the translation except the input RNA for 10 min on ice. Under these conditions, >50% of the radiolabel incorporation was associated with the phytochrome polypeptide.

The effect of phycocyanobilin addition to synthetic apophytochrome was next examined. Because *in vitro* translation produces too little protein for spectrophotometric examination, evidence for assembly of photoactive holoprotein was based on a light-dependent differential proteolysis assay. The protein conformational change that accompanies the reversible photoconversion between the Pr and Pfr forms of phytochrome can be detected by a difference in the peptide digestion patterns after brief exposure to endoproteases (2, 12). This assay is particularly sensitive because apoprotein can be labeled with [^{35}S]methionine of high specific activity by *in vitro* translation.

The time course of subtilisin proteolysis indicated that addition of phycocyanobilin led to the production of a subpopulation of more protease-resistant species compared with the untreated control samples (Fig. 3). These results are consistent with the formation of a bilin–apophytochrome adduct that confers a more compact structure to the phytochrome polypeptide. To test whether bilin addition produced photoactive holoprotein, translation mixtures were irradiated with far red or red light before subtilisin incubation. If holoprotein were present, such light treatments would be expected to produce Pr or Pfr forms, respectively. Indeed, Fig. 3 shows that light treatment altered the pattern of polypeptide fragments produced by subtilisin digestion of bilin-incubated apophytochrome. Parallel experiments with thermolysin and trypsin also showed light-dependent differences in the proteolytic fragmentation patterns for phycocyanobilin-treated apophytochrome (data not shown). By contrast, as shown in Fig. 3 for subtilisin-treated samples, no light-dependent difference in the fragmentation pattern was seen for the control samples that were not incubated with

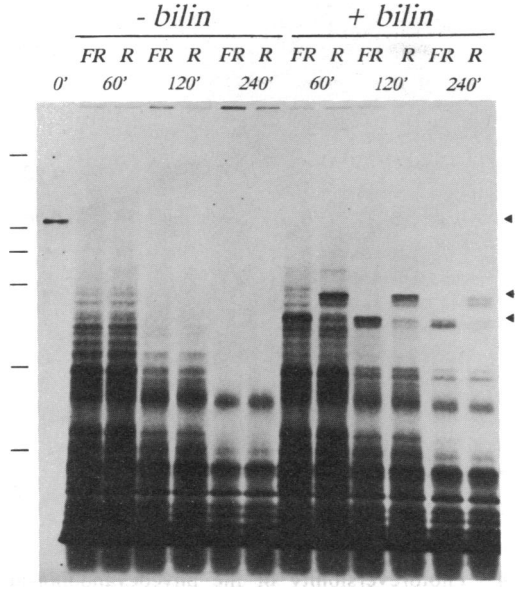

FIG. 3. Phycocyanobilin–apophytochrome adduct formation *in vitro* and its light-dependent differential proteolysis. Time courses of subtilisin proteolysis after 60, 120, and 240 min for bilin-treated (+ bilin) and control (− bilin) translation mixtures. R or FR indicate that the samples were irradiated with red or far-red light before proteolysis. No protease was added to the control translation mixture shown in the far left lane; this sample contains 1/100th of the counts that were loaded in the other lanes. The top arrowhead at right indicates migration of undigested apophytochrome. The major bilin-stabilized polypeptides seen after limited proteolysis are also indicated by arrowheads. Molecular mass markers are the same as those in Fig. 2.

bilin. The apparent molecular masses of the bilin-stabilized subtilisin breakdown products were identical to those seen for native *Avena* phytochrome preparations (data not shown). These results reflect the formation of a bilin–apophytochrome adduct, the photoexcitation of which affords a similar, if not identical, conformational change with that reported for the native holoprotein (12).

To test whether bilin treatment yielded a photoreversible protein rather than a nonnatural adduct that was differentially sensitive to red and far-red irradiation, we examined whether the effect of red irradiation could be reversed by far-red irradiation. The experiment shown in Fig. 4 indicates that the phycocyanobilin–apophytochrome adduct is photoreversible.

Formation of the apophytochrome–phycocyanobilin adduct is posttranslational and does not require ATP or other soluble cofactors. Indeed, identical results were obtained when synthetic phytochrome apoprotein was removed from most of the translation components by ammonium sulfate fractionation (data not shown). These results corroborate studies on the attachment of phycocyanobilin to apophytochrome in extracts of tetrapyrrole-deficient *Avena* seedlings (3). In contrast to these earlier studies, however, the yield of photoreversible adduct seen here appears low. Although it is difficult to quantitatively assess the yield of this reaction by limited proteolysis, we estimate that <10% of the apoprotein is converted to a photoactive adduct. This estimation is based on the observation that most bilin-treated digestion products appear the same as those of the untreated controls with only a small proportion being different (see Figs. 3 and 4). Preliminary experiments with elevated concentrations of phycocyanobilin show no obvious increase in the yield of photoreversible adduct. Other possible explanations for this low yield include the following: (*i*) phycocyanobilin is attached, but most of the adduct is not photoreversible; (*ii*) photoreversibility requires the formation of a homodimer that is not favored under our experimental conditions; and (*iii*) the apophytochrome produced in rabbit reticulocyte lysates is a mixture of denatured and native species.

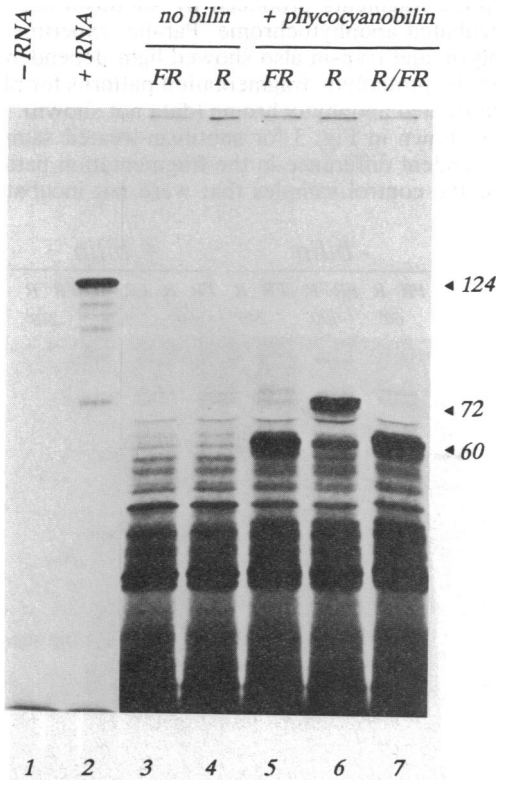

FIG. 4. Photoreversibility of the phycocyanobilin–apophytochrome adduct. This experiment was performed as in Fig. 3, except that the sample irradiated with red light (R) was divided in two, and one aliquot received an additional treatment with far-red light (R/FR); all samples were proteolyzed for 45 min (lanes 3–7). Undigested translation mixtures obtained in the absence (lane 1) or presence (lane 2) of pPC3 RNA are also shown. Identical counts were loaded in all lanes; however, the fluorograph exposures for lanes 1 and 2 were 10-fold less than those for the other lanes. Molecular mass markers are the same as those in Fig. 2. Molecular masses in kDa of major bilin-stabilized polypeptides resistant to proteolysis are indicated at right.

In conclusion, these results support the hypothesis that apophytochrome is an enzyme that catalyzes bilin attachment and holoprotein assembly. While it is possible that rabbit reticulocyte lysates contain a bilin lyase activity, there is no precedent for such an enzyme in mammalian tissue. In view of an apparent lack of an exogenous enzyme requirement, the assembly of the phytochrome holoprotein appears unique among chromoproteins that have tetrapyrrole prosthetic groups thioether-linked with the apoprotein. In this regard, the enzyme cytochrome *c* lyase is required for the formation of the thioether linkages between apocytochrome *c* and heme (13). Glazer and coworkers (14–16) reported that, although bilin attachment to purified synthetic apophycocyanin proceeded spontaneously *in vitro*, nonnatural adducts were produced, suggesting that additional enzymatic activities are also necessary to form functional phycobiliproteins. The ability of apophytochrome to self-assemble with phycocyanobilin to form a photoactive holoprotein should facilitate analysis of the structural features of the phytochrome molecule that confers photoreceptor function.

Plasmids pAP3.2 and pGP8.2-2 were kindly provided by Dr. Peter H. Quail (U.S. Department of Agriculture Plant Gene Expression Center, Albany, CA). We also thank Dr. Alexander N. Glazer (Department of Microbiology and Immunology, University of California, Berkeley) for the phycocyanobilin and Chris Bidwell for the reticulocyte lysate. This work was supported, in part, by the U.S. Department of Agriculture (GAM 8600976).

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