Subunit Interactions in the Carboxy-Terminal Domain of Phytochrome†

Michael D. Edgerton,*,‡ and Alan M. Jones

Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280

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ABSTRACT: We have produced defined fragments of the oat PhyA AP3 protein using an *in vitro* translation system and analyzed the quaternary structure of these fragments by size exclusion chromatography. Sequences between amino acids S599 and L683 are shown to dimerize by this *in vitro* assay and by a λ repressor-based *in vivo* assay. A subset of this dimerization region, V623–S673, which has previously been identified as being involved in interdomain interactions on the basis of the behavior of overlapping constructs in a λ repressor assay for protein–protein interaction, is shown by both assays to be necessary but insufficient for dimerization. Sequences between L685 and R815, which are unable to dimerize by themselves, are shown to interact with sequences between S599 and L683. Sequences E1069–Q1129, also previously suggested to be involved in dimerization, are shown here not to be required for phytochrome dimerization. These results based on an *in vitro* assay have confirmed some of the results previously obtained using an *in vivo* assay and extend these earlier results by revealing new protein–protein interactions. This dissection of sequences involved in phytochrome dimerization taken together with previous work has enabled us to propose a model for the behavior of the dimerization region where the core structure involved in dimerization is located on both sides of a region around residue 750 found at the surface.

Phytochrome is a family of photoregulatory proteins responsible for the perception of red and far red light signals (Thomas & Johnson, 1991; Quail, 1991). This chromoprotein can exist in either of two photoconvertible conformations designated Pr and Pfr. Phytochrome is synthesized in the red light absorbing conformation, Pr (λ_{max} 660 nm), and may be reversibly photoconverted to the far red light absorbing conformation, Pfr (λ_{max} 730 nm), upon absorption of red light. Photoreversible seed germination, de-etiolation, and flowering initiation have all been demonstrated (Borthwick et al., 1954; Downs, 1955, 1956; Kendrick & Kronenberg, 1986).

Phytochrome is a homodimer composed of 120–127-kDa subunits (Quail et al., 1991). Each subunit is composed of two similar sized domains. The amino-terminal, 65-kDa domain of each subunit contains a covalently bound chromophore, structures necessary for photoreversibility, and phytochromobilin lyase activity (Wahleithner et al., 1991; DeForce et al., 1991). The 55-kDa, carboxy-terminal domain contains dimerization contact sites and sequences which may be involved in light-regulated degradation of phytochrome (Jones & Quail, 1986; Shanklin et al., 1989). The two domains are joined by a proteolytically-sensitive hinge region located near residue 600 (Grimm et al., 1988; Jones & Erickson, 1989). Two similar models of phytochrome quaternary structure have been presented, both of which suggest that two amino-terminal domains protrude from dimerizing carboxy-terminal domains (Jones & Erickson, 1989; Tokutomi et al., 1989).

We are interested in identifying sequences involved in dimerization and intrasubunit interactions in the carboxy-terminal domain of phytochrome. Here we present results which confirm and extend our previous results on the identification of sequences involved in subunit interaction using a λ repressor-based *in vivo* assay for protein–protein interactions (Edgerton & Jones, 1992). We have developed a rapid assay for dimerization based on the use of *in vitro* translation and size exclusion chromatography (SEC) and have used this assay to show that sequences located between S599 and L683 of the carboxy-terminal domain of oat PhyA AP3 phytochrome are sufficient for dimer formation. A subset of these sequences, V623–S673, common to constructs which self-associate in a λ repressor-based assay for protein–protein interactions (Edgerton & Jones, 1992), is necessary for dimerization but requires adjacent sequences to dimerize. We have also identified previously undetected interactions between sequences within the carboxy-terminal domain of phytochrome. We discuss several conflicting reports concerning the location of dimerization sequences within the carboxy-terminal domain (Jones & Quail, 1986; Yamamoto & Tokutomi, 1989; Romanowski & Song, 1991; Edgerton & Jones, 1992). Our data, together with previous work, have been used to formulate a model for inter- and intrasubunit interactions within phytochrome.

MATERIALS AND METHODS

Phytochrome Constructs. Phytochrome sequences encoding oat phyA AP3 amino acids R596–Q1129 (where the initiation methionine is designated as amino acid 1) were PCR amplified out of pCIB372 (Thompson et al., 1989) using primers which placed *Bam*HI restriction sites at both ends of the coding sequences and a unique *Nhe*I site preceding an ATG initiation codon at the 5′-end of the coding sequences. The PCR product was subcloned into the *Bam*HI site of pBS(+) (Stratagene, La Jolla, CA) to produce pME/PC596–1129. Construction of the λ repressor expression vector pME10 and λ repressor/phytochrome fusion plasmids pME10/PC599-683, pME10/PC685-818, and pME10/PC488-673 is described in Edgerton and Jones (1992). For *in vitro* transcription, λ repressor and λ repressor/phytochrome sequences encoded by the ME10 series of plasmids were amplified by PCR using a primer containing a consensus T3 RNA polymerase promoter positioned 5′ of the coding sequences. The T3 RNA polymerase promoter primer (5′-ACGCGTATTTACCCTCAC-

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‡ Present address: Glaxo Institute for Molecular Biology, 14 chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland, Case postale 674.

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TAACGGGAAGCTCGAGATGAGCACAAAAAA-GAAACC-3′) was designed to conform as closely as possible to the consensus T3 RNA polymerase promoter, while being as dissimilar as possible from the T7 RNA polymerase promoter already located in pME10 (McGraw et al., 1985; Klement et al., 1990). The λ repressor/*Arabidopsis* phytochrome constructs were generated by directed cloning of sequences amplified from genomic clones of *Arabidopsis* (A.t.) *phyA*, *phyB*, and *phyC* into the *Kpn*I and *Hin*dIII sites of pME10.

In Vitro Transcription and Translation. Capped transcripts were generated using either T3 RNA polymerase (Ambion, Austin, TX) for the PCR products or T7 RNA polymerase for pME/PC596-1129 and pME/PC818-1129. The RNAs were translated in a wheat germ *in vitro* translation system (Ambion, Austin, TX) using [^{35}S]methionine as the radiolabel. Reticulocyte lysate did not translate the λ repressor fusion proteins as efficiently as did the wheat germ system.

Immunoprecipitation. The identity of the translation products was initially verified by immunoprecipitation using polyclonal antiserum NC07, raised against purified λ repressor, or the anti-phytochrome monoclonal antibody Pea-25 (Cordonnier et al., 1986). *In vitro* translation products were diluted into 600 μL immunoprecipitation buffer (20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.1% SDS (pH 7.0), 4 °C) and incubated with 50 μL of goat anti-rabbit or goat anti-mouse magnetic secondary antibodies (Advanced Magnetics, Cambridge, MA) for 2 h at 4 °C. The secondary antibodies were spun out at 12000*g* for 2 min, and increasing amounts of primary antiserum or monoclonal antibody were added. Antibody binding was allowed to proceed overnight at 4 °C. Translation products were precipitated 4 h after the addition of 200 μL of magnetic secondary body using a magnetic microcentrifuge holder (Promega, Madison, WI). The bound material was washed three times in immunoprecipitation buffer. The precipitate was then denatured in 40 μL SDS sample buffer (2% SDS, 750 mM 2-mercaptoethanol, 12% glycerol, 125 mM Tris, and 0.025% bromophenol blue (pH 6.8)), separated by SDS–PAGE, and visualized by fluorography.

Size Exclusion Chromatography. Translation products were centrifuged at 47000*g* for 15 min and then loaded onto a TSK2000 or TSK3000 size exclusion column (Beckman, Berkeley, CA) equipped with a precolumn. The columns were run at 0.4 mL/min in TSK buffer (50 mM Tris (pH 7.0), 100 mM NaCl, 5 mM 2-mercaptoethanol, 0.5 mM EDTA, and 10% glycerol) on a Shimadzu HPLC system. Fractions were collected every 200 μL, TCA-precipitated, resuspended in SDS sample buffer, and separated by SDS–PAGE. Translation products were visualized by fluorography. The size exclusion columns were calibrated with DNA (void), alcohol dehydrogenase (150 kDa), BSA (132 and 66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome C (14 kDa), all purchased from Sigma (St. Louis, MO).

λ *Repressor and β-Galactosidase Quantitation.* *Escherichia coli* strain X90K [λ200, F′{proAB, lacI^{q}1, lacZ::Tn5(Kan^{r})}, ara, Δlac, pro13, nalA, argE(am), rif, thi-1] containing pME10 or other λ repressor/phytochrome constructs was grown in media A (Miller, 1972) with varying concentrations of IPTG to an OD_{600} of approximately 0.4 as described in Edgerton and Jones (1992). Cells were either assayed directly for β-galactosidase activity as described by Miller (1972) with chloroform and SDS lysis or 1 mL of culture was spun down for 1 min at 12000*g* and resuspended in 200 μL of SDS sample buffer and denatured at 65 °C for 10 min for quantitation by immunoblotting. Immunoblot quantitation of λ repressor fusions and β-galactosidase was done simultaneously by separating 0.05 OD_{600} units of denatured cells on 15% SDS–PAGE and transferring the proteins to nitrocellulose filters. The filters were blocked overnight in TBS (50 mM Tris, 150 mM NaCl, and 0.05% NaN_3) with 1% Carnation nonfat dry milk at 25 °C. The blocked filters were incubated with affinity-purified, anti-repressor antiserum NC07 and anti-β-galactosidase monoclonal antibody (Promega, Madison, WI) simultaneously in TBS plus 0.1% BSA overnight at 4 °C. Repressor was detected using the ECL system (Amersham, Arlington Heights, IL) with goat anti-rabbit secondary and rabbit anti-goat horseradish peroxidase conjugated tertiary antibodies. β-Galactosidase was then detected using alkaline phosphatase conjugated goat anti-mouse secondary antibodies. Quantitation was performed on a Molecular Dynamics scanning densitometer. Serial dilutions of the sample were used to ensure linearity of response for densitometric quantitation.

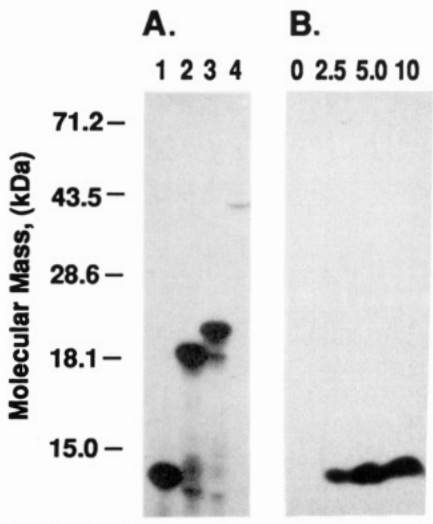

FIGURE 1: Production of defined phytochrome segments by *in vitro* translation. (A) ME10 (λcI M1-92 plus 27 polylinker-encoded amino acids) (lane 1), ME10/PC599-685 (ME10 fused to phytochrome sequence 599-685) (lane 2), ME10/PC685-818 (lane 3), and phytochrome R596–Q1129 (lane 4) proteins were produced in a wheat germ *in vitro* translation system and analyzed by fluorography after electrophoresis on a 15% SDS–polyacrylamide gel. (B) Immunoprecipitation of the ME10 protein with 0, 2.5, 5.0, and 10 μL of NC07 anti-repressor immunoglobulins.

RESULTS

Experimental Approach. We produced a series of defined phytochrome fragments and ME10 fusion proteins in an *in vitro* translation system and analyzed the quaternary structure of these proteins by size exclusion chromatography (SEC). By studying the behavior of these protein fragments, we were able to circumvent problems that previous workers have encountered with the precise identification of proteolytic fragments (Jones & Quail, 1986) and also to sort out complex interactions occurring between different sections of the protein. However, as will be discussed in greater detail, this widely used *in vitro* approach has its own limitations.

Figure 1a shows a fluorograph of radiolabled ME10 protein (λ repressor residues M1–R92 plus 27 residues encoded by polylinker sequences), two λ repressor/phytochrome fusion proteins (λcI M1–R92/phytochrome S599–L683 and λcI M1–R92/phytochrome L685–R815), and phytochrome R596–Q1129 (the carboxy-terminal domain) produced in a wheat germ *in vitro* translation system. This film was overexposed

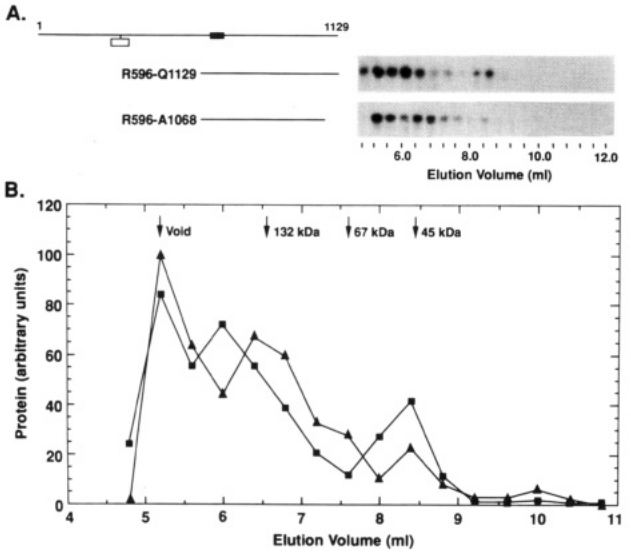

FIGURE 2: Size exclusion chromatography elution profile for phytochrome sequences R596–Q1129 and R596-A1068. (A) Odd-numbered fractions from a TSK3000 size exclusion column were analyzed by fluorography after electrophoresis on a 10% SDS–polyacrylamide gel. (B) Fluorographs from duplicate experiments were quantitated by densitometry, averaged, and plotted as the amount of protein (arbitrary units) vs elution volume for R596–Q1129 (■) and R596–A1068 (▲). Elution volumes of standards are indicated by arrows. Open box represents position of chromophore. Filled box represents protein–protein interaction site located by λ repressor assay (Edgerton & Jones, 1992).

to make two points. First, only one major translation product was produced in each reaction. Second, the λ repressor fusion proteins consistently translated with much higher efficiencies than nonfusion phytochrome proteins (compare lanes 1–3 with lane 4 in Figure 1a) and were present at much higher molar concentrations than the nonfusion proteins. The identities of the proteins shown in Figure 1a were confirmed by molecular mass measurements, by immunoprecipitation with anti-repressor and/or anti-phytochrome antisera, and by immunoblotting using anti-repressor and/or anti-phytochrome antisera. Figure 1b shows the results of a representative immunoprecipitation experiment for the ME10 protein.

In Vitro Translated Carboxy-Terminal Domains Dimerize in a Native-like Manner. Figure 2a shows a size exclusion column elution profile for the carboxy-terminal domain of phytochrome, R596–Q1129, produced by *in vitro* translation. In this experiment, 25 μL of a translation reaction was subjected to SEC (TSK3000 column). Odd-numbered fractions were TCA-precipitated, resuspended in SDS sample buffer, and analyzed by SDS–PAGE and fluorography. Figure 2b shows the results of densitometric quantitation of the SEC elution profile for replicate experiments. Three distinct protein populations were seen: One which eluted at the void volume, a second with an apparent molecular mass of about 200 kDa, and a third population with an apparent molecular mass of about 50 kDa. These three populations of R596–Q1129 were, respectively, high-order aggregates, tightly associated nonglobular dimers, and globular monomers. Our reasoning for this interpretation is as follows: (1) Since the R596–Q1129 protein has a calculated molecular mass of 58.6 kDa, the peak eluting at 50-kDa apparent molecular mass can only be interpreted as a stable population of globular monomers. (2) The 200-kDa peak elutes from the size exclusion in a manner similar to that reported in elution profiles for dimeric carboxy-terminal domains derived from proteolytic cleavage of native phytochrome. Two studies have shown that the carboxy-terminal domain has an apparent molecular mass between 250 and 150 kDa and a frictional ratio of 1.44 (Vierstra & Quail, 1985; Jones & Quail, 1986). Furthermore, phytochrome R596–Q1129 produced in a baculovirus expression system elutes from the same size exclusion column used in this study as a *single* peak of 200 kDa (M. D. Edgerton, unpublished data). Thus, the 200-kDa peak is composed of a stable population of native-like dimeric carboxy-terminal domains. (3) The void volume peak most likely represents phytochrome which has aggregated due to misassembly. Recently, we (Edgerton et al., 1993) and others (Grimm et al., 1993; Mummert et al., 1993) demonstrated the involvement of the chaperonins GroEL/S in phytochrome folding. This aggregation is probably due to limiting amounts of a chaperone present in the wheat germ translation system. Lagarias and Lagarias (1989) have similarly been unable to produce completely native populations of phytochrome in reticulocyte lysates.

The dimeric interactions observed in this assay are extremely tight. By comparison, the R596–Q1129 protein migrates with the same apparent molecular weight as the carboxy-terminal domain produced by proteolysis of the native molecule. It is known that the proteolytically derived fragment dimerizes at a subunit concentration of 1.6 μM (Jones & Quail, 1986), indicating that the R596–Q1129 protein was present at a concentration greater than or equal to the dissociation constant since a dimeric population was also observed (Ackers, 1970; Valdes & Ackers, 1979). Therefore, it is possible to approximate a maximum value for a dissociation constant for the carboxy-terminal domains. Since typical *in vitro* translation reactions produce 1–10 ng of protein (Struhl, 1989), we calculate the maximum concentration for the 58.6-kDa R596–Q1129 protein to be less than 500 pM in a 50-μL reaction. Thus, a rough maximum value for a dissociation constant can be considered to be no greater than 500 pM. While we realize that this can only be considered a crude estimate, this low dissociation constant is consistent with observations that only strong denaturants or high salt concentrations are able to produce phytochrome monomers (Jones & Quail, 1986; Yamamoto & Tokutomi, 1989; Choi et al., 1990). No other estimate for a dimerization constant has been published to date.

Sequences between E1069 and Q1129 Are Not Required for Dimerization. In a previous study, we deduced that sequences between N1049 and Q1129 could be involved in dimerization. We identified a potential amphipathic α-helix, designated "helix d", located between N1094 and G1109 within this region, which we suggested could be involved in dimerization (Edgerton & Jones, 1992). To test this hypothesis, we truncated the carboxy-terminal domain at A1068 to produce a protein, R596-A1068 (52 kDa), that lacks "helix d". Figure 2 shows that the R596–A1068 protein elutes from the size exclusion column in three populations, much like the R596–Q1129 protein. There is an aggregated population eluting at the void volume, a nonglobular dimeric population eluting at roughly 150 kDa, and a globular monomeric population eluting at about 50 kDa. The slightly smaller size of the R596–A1068 dimer relative to the R596–Q1129 dimer indicates that sequences carboxy of A1068 are either (1) located toward the periphery of the molecule, such that removing them reduces the molecular radius of the protein, or (2) removal of E1069–Q1129 slightly increases the dissociation constant for dimerization. Proteolytic analysis (Grimm et al., 1988) and binding studies with monoclonal antibody Z-4A5 (Schneider-Poetsch et al., 1989) have shown

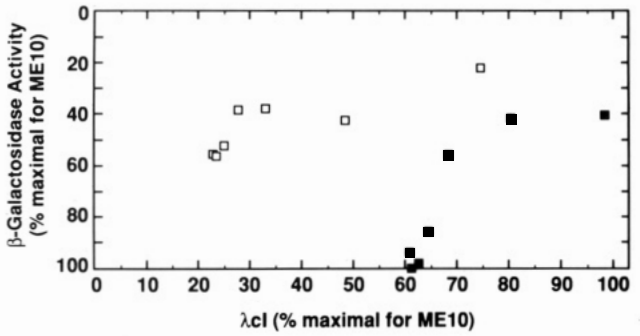

FIGURE 3: *In vivo* repressor activity vs repressor concentration. Activity of ME10 (truncated λ repressor, λcI 1-92) (■) and ME10/PC599-683 (□) protein measured as the decrease in λP_R-regulated β-galactosidase synthesis is plotted vs amount of repressor amino-terminal domain determined by quantitative immunoblotting with anti-repressor antiserum NC07. Data are from duplicate experiments. Error is approximately ±15%. This assay is described in more detail in Materials and Methods and in Edgerton and Jones (1992).

that this region of the protein is near the surface. Therefore, it is reasonable to expect that the reduced apparent molecular mass of the R596–A1068 protein reflects a decrease in the molecular radius of phytochrome rather than an increase in the dissociation constant. Nonetheless, it is clear that sequences between E1069 and Q1129 are not necessary for dimerization of the carboxy-terminal domain.

Sequences Involved in Dimerization Are Located between S599 and L683. Phytochrome sequences between V623 and S673 were previously deduced to be necessary for protein–protein interactions on the basis of their behavior in a λ repressor assay for such interactions. In this assay system, the carboxy-terminal domain of λcI, which contains most of the dimeric contacts in the intact repressor (Pabo et al., 1979; Gimble & Sauer, 1989), was replaced with segments from phytochrome. Because λ repressor binds DNA as a dimer (Chadwick et al., 1971), the improved function for a given repressor/phytochrome fusion indicated that the heterologous segments were able to interact, substituting for the native λ repressor dimerization sequences. Four overlapping segments, containing sequences in common between V623 and S673, were all shown to partially restore repressor function (Edgerton & Jones, 1992). We have further examined the role of sequences centered about V623 and L673 in dimerization using both *in vivo* and *in vitro* assays. The ability of the ME10/PC599-683 fusion protein was directly compared to the ability of the ME10 protein to repress β-galactosidase synthesis by quantitative immunoblotting. As shown in Figure 3, ME10/PC599-683 was able to form dimers at much lower protein concentrations than ME10, consistent with our previous conclusions (Edgerton & Jones, 1992). The ability of proteins containing these sequences to dimerize was also assessed *in vitro* by analyzing SEC elution profiles of both a small carboxy-terminal protein, R596–R815, and the λ repressor/phytochrome fusion protein, ME10/PC599-683.

As shown in Figure 4, phytochrome fragment R596–R815 (24 kDa) elutes from the size exclusion column in two populations, one with an apparent molecular mass of roughly 100 kDa and a second with an apparent molecular mass of about 50 kDa. No R596–R815 protein eluted at the void volume. ME10/PC599-683 (21 kDa) also elutes from the size exclusion column in two populations, one with an apparent molecular mass of about 90 kDa and a second with an apparent molecular mass of 40 kDa. Again, no protein was observed in the void volume. ME10 (13 kDa) elutes as a single population with an apparent molecular mass of about 15 kDa,

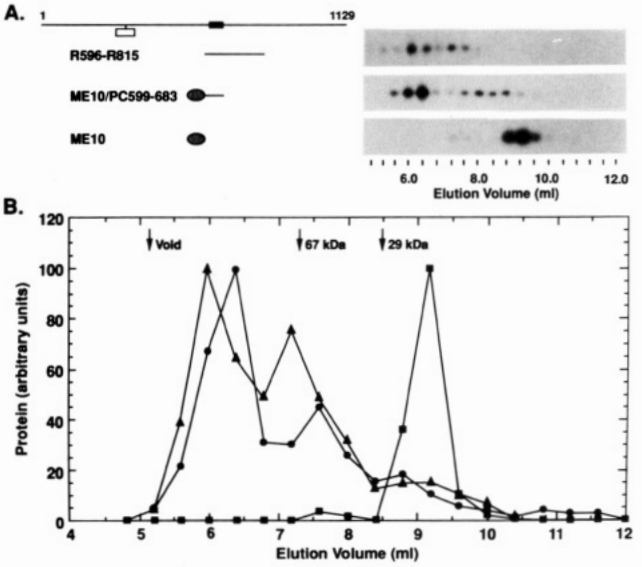

FIGURE 4: Size exclusion chromatography elution profile for phytochrome R596–R815, ME10 (truncated λ repressor, λcI 1-92), and ME10/PC599-683 (ME10 fused to phytochrome sequence 599-683). (A) Odd-numbered fractions from a TSK2000 size exclusion column were analyzed by flourography after electrophoresis on a 15% SDS–polyacrylamide gel. (B) Fluorographs from duplicate experiments were quantitated by densitometry, averaged, and plotted as the amount of protein (arbitrary units) vs elution volume: R596–R815 (▲), ME10/PC599-683 (●), and ME10 (■). Elution volumes of standards are indicated by arrows. Open box represents position of chromophore. Filled box represents protein–protein interaction site located by λ repressor assay (Edgerton & Jones, 1992).

consistent with the expected monomeric behavior of the amino-terminal domain of λ repressor at concentrations of less than 3 μM (Weiss et al., 1987).

The simplest interpretation of the behavior of both R596–R815 and ME10/PC599-683 in SEC, on the basis of their expected nonglobular shapes (Jones & Quail, 1986), is that they have folded into noninteracting stable pools of dimers and monomers. While tetramers of both phytochrome and λ repressor have been observed, they have only been observed at much higher protein concentrations and unusual salt concentrations (Choi et al., 1990; Baink et al., 1993; Brack & Pirrotta, 1975). However, we do not exclude the possibility of tetramer/dimer formation. On the basis of either interpretation of this *in vitro* SEC data in addition to the *in vivo* data (Figure 3), we conclude that phytochrome sequences between S599 and L683 are capable of mediating dimerization, confirming our previous conclusions based on the *in vivo* assay (Edgerton & Jones, 1992).

Sequences between V623 and S673, While Necessary, Are Not Sufficient for Dimerization. Three λ repressor/phytochrome constructs, encoding sequences homologous to oat PhyA V623–S673, were made from the *Arabidopsis phyA*, *phyB*, and *phyC* genes (Sharrock & Quail, 1989). We focused attention on these 50 residues because proteins containing these residues in common all improved repressor activity *in vivo* (Edgerton & Jones, 1992). *Arabidopsis* PhyA, PhyB, and PhyC are respectively 82, 56, and 58% identical to the oat PhyA sequences between V623 and S673, and when conservative replacements are considered, they are, respectively, 92, 82, and 82% similar. Each of these ME10/*Arabidopsis* phytochrome constructs was assayed for its ability to self-associate both *in vivo* and *in vitro*.

Figure 5a shows the results of an *in vivo* protein–protein interaction assay for each of the ME10/*Arabidopsis* constructs. Repressor activity, measured as inhibition of λP_R-

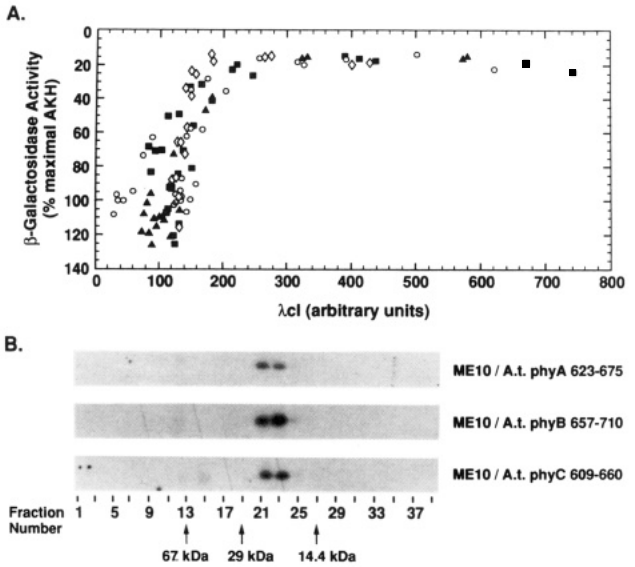

FIGURE 5: *In vitro* and *in vivo* assays for dimerization for *Arabidopsis* PhyA, PhyB, and PhyC sequences homologous to oat PhyA V623-S673. (A) *In vivo* repressor activity vs repressor concentration. Activity of ME10 (■), ME10/A.t.PhyA V623–E675 (O), ME10/A.t.PhyB V657–A710 (▲), and ME10/A.t.PhyC V609–V660 (◇) proteins measured as decreases in λP_R-regulated β-galactosidase synthesis is plotted vs the amount of repressor amino-terminal domain determined by quantitative immunoblotting with anti-repressor antiserum NC07. Data shown is from three (ME10 and ME10/A.t.PhyA V623–E675) or two experiments (ME10/A.t.PhyB V657–A710 and ME10/A.t.PhyC V609–V660). Maximal AKH in the ordinate label represents the averaged β-galactosidase activity for ME10/A.t.PhyA V623–E675 at zeroo induction. (B) ME10′/A.t.PhyA V623–E675, ME10′/A.t.PhyB V657–A710, and ME10′/A.t.PhyC V609–V660 were translated *in vitro* and analyzed by size exclusion chromatography on a TSK2000 size exclusion column followed by electrophoresis on a 15% SDS–polyacrylamide gel and fluorography. ME10′ is a deletion mutant of ME10 lacking repressor amino acids 2–7, which are involved in DNA binding (Clarke et al., 1991).

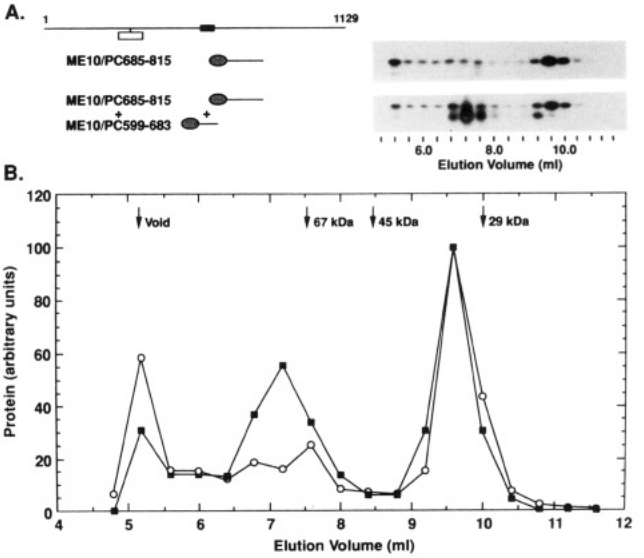

FIGURE 6: Size exclusion chromatography elution profile for ME10/PC685-815 (truncated λ repressor fused to phytochrome sequence 685-815) translated with (■) or without ME10/PC599-685 (O). (A) Odd-numbered fractions eluting from a TSK3000 size exclusion column were analyzed by fluorography after electrophoresis on a 10% SDS–polyacrylamide gel. (B) Fluorographs from duplicate experiments were quantitated by densitometry, averaged, and plotted as the amount of protein (arbitrary units) vs elution volume. Elution volumes of standards are indicated by arrows. Open box represents position of chromophore. Filled box represents protein–protein interaction site located by λ repressor assay (Edgerton & Jones, 1992).

regulated β-galactosidase synthesis, is plotted against repressor protein concentration measured by quantitative immunoblotting. In each case, activity of the repressor/phytochrome constructs is not seen to significantly differ from activity of the ME10 protein, suggesting that these minimal constructs are not able to dimerize. Figure 5b shows SEC elution profiles for these constructs. The ME10/*Arabidopsis* phytochrome constructs (16 kDa) all eluted from the size exclusion column as globular monomers with an apparent molecular mass of about 20 kDa, confirming the *in vivo* results. Thus, sequences between V623 and S673 previously identified by overlap in the *in vivo* assay are not sufficient for dimerization in either *in vitro* or *in vivo* assays. Given that R596–R815 and ME10/PC599-683 have been shown to dimerize, this suggests that sequences on at least one side and possibly both sides of V623–S673 are needed for dimerization to occur. We note that sequences between D606 and M622, just outside the region defined by overlap, are highly conserved and could be required for dimerization.

Interaction between S599–L683 and L685–R815. Figure 6 shows an SEC elution profile for ME10/PC685-815 (27.5 kDa). Two populations are observed for this protein: One population of aggregated protein elutes with the void volume, and the second population migrates as an extended monomer with an apparent molecular mass of 30–40 kDa. This is consistent with the results of our *in vivo* analysis of the ME10/PC685-815 protein, in which this protein was shown to be unable to suppress λP_R-driven β-galactosidase synthesis significantly better than the ME10 protein (Edgerton & Jones, 1992).

When translated alone, the fusion protein ME10/PC685-815 aggregated significantly. Such behavior was not observed for the R596–R815 and ME10/PC599-683 proteins (Figure 3), suggesting that aggregation of sequences between L685 and R815 is suppressed when sequences between S599 and L683 are present. There are three possible reasons for this: (1) S599–L683 interacts with L685–R815 to cover a region of the protein at which it aggregates; (2) S599–L683 interacts with L685–R815 to stabilize the folded structure of the protein; or (3) S599–L683 could serve as a template which L685–R815 requires to achieve its properly folded state. To address this issue, we cotranslated ME10/PC599-683 with ME10/PC685-815. As shown in Figure 5, two changes in the ME10/PC685-815 elution profile occurred. First, the percentage of aggregated ME10/PC685-815 decreased. Second, a population which migrated with an apparent molecular weight of about 90 kDa, the same apparent molecular weight as the ME10/PC599-683 dimer, appeared. For these experiments, translations were run with equal amounts of the two RNAs at concentrations well below saturation of the translation reaction. Single and double translation reactions (elution profiles) were exposed on the same film to show that total protein yields were similar for both reactions.

The reduced aggregation and the appearance of a new peak demonstrate that sequences between L685 and R815 are able to dimerize either directly or by interacting with sequences between S599 and L683 at nanomolar or lower concentrations. This new peak could be composed of either ME10/PC685-815 dimers or a complex of dimeric ME10/PC599-683 and two additional ME10/PC685-815 proteins. In the former possibility, sequences between S599 and L683 would have to serve as a folding template, which interacts with a folding intermediate of ME10/PC685-815 to assist the molecule in attaining its native dimeric conformation. In the latter possibility, a globular complex is formed between two dimerizing ME10/PC599-683 molecules and two ME10/

PC685-818 molecules. Such a complex would have a total molecular mass of 94 kDa, a molecular radius similar to that of ME10/PC599-683, and a globular shape in which the unoccupied volume of the extended ME10/PC599-683 dimer is filled with two additional ME10/PC685-815 proteins. Such an interpretation is consistent with our observations that the ME10/PC685-815 protein is not able to dimerize either *in vitro* or *in vivo* and our observations that both the ME10/PC599-683 and R596–R815 proteins are asymmetric.

DISCUSSION

When the atomic coordinates of a protein are not available, dissecting the interactions within and between domains (independent folding units) is not a simple matter and it must rely on the interpretation of data obtained by various methods in more than one approach. Two approaches were used to identify protein interactions in phytochrome: (1) Jones and Quail (1986) used intact phytochrome in the Pfr form as the starting material and digested it to produce a large set of fragments. These fragments were analyzed by SEC and cross-linkers. (2) The present study and Edgerton and Jones (1992) used specific sequences of phytochrome as the starting material and examined the interactions using SEC and a λ repressor-based assay. Each approach with its various assays has its advantages as well as its limitations. The former approach can reveal native interactions formed within the intact molecule (Taniuchi et al., 1967). However, identification of these putatively native structures depends on having specific reagents to sort the many fragments, and it is incorrect to assume that native structure is maintained or represented collectively by the various fragments. In the later approach, the complexity is reduced because only specific peptide fragments are expressed and analyzed. However, not all primary sequences fold into stable structures outside the context of the entire protein, nor is it correct to assume that folded structures are representative of a domain of the intact protein. A third approach which has not yet been utilized is linker scan and point mutagenesis coupled with assays of dimerization. This approach is limited by the effects of mutation on the stability of folded and intermediate structures and on the folding pathways.

Considered on their own merit, the data obtained by the first and second approaches discussed above lead to different conclusions on the location of the dimerization region in phytochrome. Cleavage of the intact molecule at approximately residue 750 [site determined by Grimm et al. (1988)] released an 80-kDa monomer and a 37-kDa dimer (Jones & Quail, 1986), suggesting that the dimerization region is carboxyl to residue 750. Parker et al. (1991) came to a similar conclusion using a structure prediction algorithm. In contrast, our data indicate that the phytochrome sequence between 599 and 683 appears dimeric in two types of assays, suggesting that the dimerization region is amino-terminal to residue 750. Furthermore, by these methods, the region between 685 and 815 is incapable of dimerizing unless the sequence between 599 and 683 is present, either in cis or in trans.

Taken together, these results support the conclusion that dimerization within the carboxy-terminal part of phytochrome is complex and involves multiple interactions between and within the subunits. As shown in Figure 7, we have indicated strong interactions detected by the *in vitro* and *in vivo* assays. The interaction observed between residues 599 and 685, however, is not found in the intact Pfr molecule, based upon the work of Jones and Quail (1986). This may mean that within the fully folded phytochrome protein this domain is

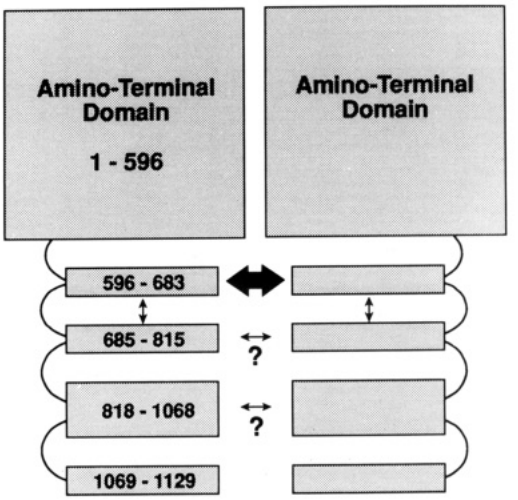

FIGURE 7: Schematic model for subunit interactions occurring in the carboxy-terminal domain of phytochrome based on SEC of the translated phytochrome sequences. Interactions between different regions of the carboxy-terminal domain are indicated by arrows. The size of the arrow indicates the relative strength of the interaction as observed in the *in vitro* assay. Speculative interactions are indicated by question marks. The apparent molecular mass of a protein when determined by small-zone size exclusion chromatography is dependent on shape, molecular radius, and in the case of self-associating proteins, protein concentration (Ackers, 1970; Valdes & Ackers, 1979). Since the maximum concentration for all of the test proteins in this study is 10 nM, this *in vitro* assay detects only strong interactions. Interactions which occur with micro- or millimolar dissociation constants are unlikely to be detected by this assay.

insufficient to tether the two subunits together, although this domain may have an important dimerization role for a folding intermediate. This is consistent with our observation that this region (599–683) decreases the amount of aggregation and increases the amount of folding of another domain (685–815). If regions on both sides of residue 750 are involved in dimerization in the intact molecule, then they must have different relative contributions in truncated forms of phytochrome. The data support the conclusion that the amino-terminal chromophore domain must destabilize dimerization of the 599–685 domain when phytochrome is truncated at approximately residue 750. A similar suggestion has been made previously by Yamamoto and Tokutomi (1989).

A more speculative interpretation of these conflicting results is that the *in vitro* and *in vivo* assays of phytochrome fragments are mapping protein–protein interactions that occur in the Pr form of phytochrome (this work; Edgerton & Jones, 1992), whereas the works based upon digestion of intact phytochrome (Jones and Quail, 1986) are reporting the Pfr conformation for this region. The latter studies used Pfr as the starting material, and it is possible that, in the former studies, phytochrome fragments adopt a Pr conformation in the *in vitro* translation and λ repressor-based assays. A conformational change has been mapped to residue 750 located between these two putative dimerization regions.

The dimeric interaction between 599 and 685 is not artifactual and represents a plausible structure within fully folded phytochrome or its folding intermediates. The possibility that the 599–685 domain forms hydrophobic interfaces upon which only nonspecific associations occur is unlikely because stable dimeric pools are observed at very low concentrations. This indicates that (1) the dimeric interaction is extremely tight with a K_d at or below 1 nM and (2) that these dimeric structures are not in equilibrium with monomers and high-order aggregates. Nonspecific associations are

typically weaker and result in many more aggregation and conformation states.

Due to the size of the phytochrome polypeptide and the complexities of its subunit interaction, future studies on phytochrome structure will need to be based upon the crystal structure. Even the genetic approach discussed above will require atomic coordinates to direct the design of informative mutations and to interpret the results.

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