Quaternary Structure of 124-Kilodalton Phytochrome from *Avena sativa* L.†

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ABSTRACT: We present three lines of evidence that etiolated *Avena* phytochrome with a monomeric molecular mass of 124 kilodaltons (kDa) is a dimer in solution. First, data obtained by utilizing low-speed sedimentation equilibrium centrifugation indicate that, over the full concentration range examined (0.1–0.35 mg/mL), phytochrome behaves as a single, uniform population with a calculated molecular mass of 253 kDa. When 1 M NaCl is present, the population becomes heterogeneous with an average molecular mass of 155 kDa, indicating partial subunit dissociation. Second, when phytochrome over a concentration range of 6–66 μg/mL is covalently cross-linked with glutaraldehyde or one of a family of imido esters, the major product has an apparent molecular mass of approximately 225 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Third, when a sample of phytochrome containing a proportion of cross-linked products is chromatographed on a size-exclusion column under nondenaturing conditions, cross-linked and un-cross-linked molecules comigrate. Data from size-exclusion chromatography (SEC) provide evidence that both the intact dimer and the dissociated subunits have an elongated shape. These data indicate further that dimerization probably involves some ionic bonds and that if disulfide bridging is involved it does not account exclusively for the dimer configuration. We also present evidence that the contact site(s) between the monomers lie(s) within 42 kDa from the carboxyl terminus. First, when phytochrome is digested with trypsin and subjected to SEC under nondenaturing conditions, all peptides containing the carboxyl-terminal 42 kDa migrate as species with molecular masses 2–3 times their monomeric masses calculated from SDS–PAGE. In contrast, tryptic peptides derived from the remaining 82-kDa, amino-terminal portion behave as globular monomers. Second, glutaraldehyde cross-links the 42-kDa fragment-containing peptides at ratios of glutaraldehyde to phytochrome 2–3 times lower than for those peptides lacking the 42-kDa fragment. The higher efficiency with which these carboxyl-terminal peptides are cross-linked is interpreted to indicate that these domains are adjacent to each other in the native dimer.

Phytochrome is a plant photoreceptor which is known to control many aspects of development, e.g., shoot growth, greening, flowering, leaf abscission (Shropshire & Mohr, 1983). The molecule contains a chromophore and, by absorption of red (665-nm) or far-red (730-nm) light, is interconvertible between two forms, a red-absorbing form (Pr)[1] and a far-red-absorbing form (Pfr). The biologically active form, Pfr exerts its action by altering specific gene expression (Quail, 1984; Tobin & Silverthorne, 1985), but the exact mechanism involved is unknown.

One part of our approach to understanding this mechanism is to study the structure of the molecule and attempt to assign functions to its domains. The quaternary structure of 120-kDa, rye phytochrome was first predicted by Gardner et al. (1971) to be dimeric. They demonstrated that this molecular species behaves as an entity with an apparent molecular mass of 400 kDa in size-exclusion chromatography (SEC) and has a sedimentation coefficient of 9 S by rate zonal centrifugation, corresponding to a molecular mass of approximately 180 kDa (Gardner et al., 1971; Rice & Briggs, 1973; Correll et al., 1968). They reasoned that an asymmetrical dimer of two 120-kDa subunits would be consistent with these observations. However, these workers inadvertently based their predictions on what were most likely partially degraded, rye phytochrome preparations (Kerscher & Nowitzki, 1982). In addition, the analytical methods used are dependent on molecular shape, thus precluding definitive conclusions regarding quaternary structure. Hunt and Pratt (1980) used sedimentation equilibrium centrifugation, a shape-independent method, to determine the structure of 118/114-kDa phytochrome from oat. They too concluded that phytochrome was dimeric. However, because it was later determined that the phytochrome analyzed was partially degraded (Vierstra & Quail, 1982), it remained possible that the degraded molecule had lost domains involved in quaternary structure or had dimerized artifactually as a result of the proteolysis. Recently, Lagarias and Mercurio (1985) used SEC to characterize the structure of undegraded, 124-kDa phytochrome from oat and reported that the photoreceptor has an apparent molecular mass of 350 kDa under nondenaturing conditions. Because this method yields results that are dependent on the shape of the molecule, the data provided are insufficient to unequivocally support the conclusion drawn that phytochrome is a homodimer. An elongated monomer or a globular trimer are equally valid structures given the reported data.

We have reevaluated the quaternary structure of phytochrome, addressing the limitations of these past studies. We have used three independent methods to assay the structure

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[1] Abbreviations: $\Delta\Delta A$, difference in absorbance at 665 and 730 nm taken from the phytochrome difference spectrum (Pr − Pfr); DMA, dimethyl adipimidate; DMP, dimethyl pimelimidate; DMS, dimethyl suberimidate; f/f_0, frictional ratio; G/P ratio, glutaraldehyde to phytochrome mass ratio; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; Pr, red-absorbing phytochrome; Pfr, far-red-absorbing phytochrome; SAR, specific absorbance ratio A_{666}/A_{280} of the Pr form; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; kDa, kilodalton(s); TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; DTT, dithiothreitol.

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of undegraded, 124-kDa, oat phytochrome over a wide concentration range. In addition, we have used size-exclusion chromatography to examine the shape of phytochrome and its subunits, as well as the type of interaction maintaining the association.

Observations that the carboxyl half of proteolytically cleaved phytochrome from oat (Jones et al., 1984) and zucchini (Vierstra & Quail, 1985) behaved as an aggregate in SEC under nondenaturing conditions have been interpreted to indicate that the contact site(s) for dimerization of the native molecule lie(s) somewhere within 55 kDa from the carboxyl terminus. Although these results are strongly suggestive, some features of the experiments render them less than conclusive. First, the data were obtained with crude or only partially purified preparations, leaving open the possibility of abnormal migration of peptides in SEC due to interactions with other components in the extracts. Second, because the fractions were not monitored for potential degradation after separation in the first dimension on SEC, the possibility that postcolumn degradation generated at least some of the peptides observed in the second dimension on SDS–PAGE cannot be totally excluded. Third, the possibility that the proteolytic cleavage of the native molecule secondarily induced the aggregation of the 55-kDa fragments has not been ruled out by the data. Here we address these previous shortcomings and provide evidence from two independent sets of experiments with purified phytochrome verifying the carboxyl domain location of the contact site(s). In addition, we extend the previous studies by providing mapping data that narrow the potential location of this (these) site(s) within the carboxyl domain.

Materials and Methods

Plant Material and Chemicals. Garry oat (*Avena sativa* L.) seeds were purchased from Olds Seed Co., Madison, WI. They were sown on wetted vermiculite and grown for 3.5 days in the dark at 26 °C. Shoots were harvested by severing below the mesocotyl node and stored at 4 °C in the dark for up to 3 days. Dimethyl suberimidate (DMS), dimethyl pimelimidate (DMP), dimethyl adipimidate (DMA), and immobilized TPCK–trypsin were purchased from Pierce Chemical Co., Rockford, IL. Acrylamide, bis(acrylamide), and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Chemical Co., Richmond, CA. SDS was further purified by recrystallization in water/ethanol (Hunkapiller et al., 1983). Coomassie blue linked standards purchased from BRL, Bethesda, MD, were used in SDS–PAGE for calibration. Glutaraldehyde, in the highest available purity, was purchased from Sigma Chemical Co. (St. Louis, MO) and stored at −5 °C in sealed ampules. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), lysine, anti-rabbit immunoglobulin (Ig) prepared in goat, anti-mouse Ig prepared in rabbit, and all the molecular weight standards listed under HPLC were also purchased from Sigma Chemical Co. Rabbit polyclonal and mouse monoclonal Ig's directed against 124-kDa phytochrome were prepared as previously described (Vierstra & Quail, 1983b; Daniels & Quail, 1984). The water used in HPLC was purified by Milli Q (Millipore Corp., Bedford, MA) and filtration through 0.22-μm nitrocellulose filters.

Phytochrome Purification. Phytochrome was purified from 1 kg of oat shoots by the methods of Vierstra and Quail (1983a) except for the following modifications. EDTA in all buffers previously used in the hydroxyapatite chromatography was replaced with 10 μM $CaCl_2$. The pooled fractions of phytochrome from the hydroxyapatite chromatography were not concentrated by ammonium sulfate before being loaded onto the affigel blue column. All manipulations with phytochrome described under Materials and Methods were performed under a dim green safelight unless otherwise noted.

Sedimentation Equilibrium Centrifugation. Phytochrome (200 μg/mL) of high purity (SAR = 1.11) in 100 mM NaP_i, pH 7.8, and 1 mM EDTA was loaded into a double-sectored Kelvlar cell as Pr under dim green light. The height of the sample column was 4.5 mm. An An-F rotor was used in a Beckman (Arlington, CA) Model E analytical centrifuge to sediment the phytochrome samples at 4000 rpm, 5 °C. Equilibrium (48 h) and the approach to equilibrium were monitored at 280 nm with scanning optics. The molecular weight (M_r) was calculated by using the equation:

$$M_r = \frac{2RT\mathrm{d}(\ln c)}{(1 - \nu\rho)\omega^2 \mathrm{d}x^2}$$

where R = the gas constant (ergs per degree kelvin per mole), T = the absolute temperature (degrees kelvin), ν = the partial specific volume of phytochrome (milliliters per gram), ρ = the density of buffer (grams per milliliter), ω = the angular velocity (radians per second), c = the absorbance at 280 nm, and x = the distance from the rotor center (centimeters). The partial specific volume, 0.738 mL/g, was calculated by the method of Cohn and Edsall (1943) using the amino acid sequence derived from sequence analysis of phytochrome cDNA clones (Hershey et al., 1985). This value is similar to that value which can be calculated from published amino acid compositions of 124-kDa, oat phytochrome (Litts et al., 1983; Vierstra & Quail, 1983). The molecular mass of phytochrome was analyzed by this equilibrium centrifugation procedure 6 times utilizing different samples without NaCl and 2 times with NaCl.

Cross-Linking Phytochrome. Phytochrome (SAR = 0.85) was cross-linked with glutaraldehyde by using the following method. Phytochrome and a freshly opened ampule of glutaraldehyde were diluted with 100 mM NaP_i, pH 7.8, 1 mM EDTA, and 5% glycerol to 6–66 μg/mL and 0.02–2 mg/mL, respectively. The glutaraldehyde, phytochrome as Pfr, and additional buffer were immediately added together into an Eppendorf tube to make the indicated ratios (reaction volume was typically 60 μL), mixed, and incubated on ice for 1–1.5 h in the dark. The reaction was stopped by adding 2 μL of 1 M lysine. The total reaction mixture was diluted with electrophoresis sample buffer, and aliquots were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). When phytochrome was cross-linked with imido esters (DMS, DMP, or DMA), the same procedure was followed except that the buffer for the cross-linking reagents was 100 mM borate, pH 10, and 1 mM EDTA. The pH of the reaction was 9.2. Since the half-lives of these cross-linking reagents are short, all additions of the cross-linking reagents to phytochrome were performed immediately after they were dissolved.

Trypsin Digestion. Phytochrome samples (Pfr, SAR = 0.85) in 100 mM NaP_i, pH 7.8, 1 mM EDTA, and 5% glycerol, typically 200 μL (0.2 mg/mL), were loaded into a 5-mL syringe barrel, plugged with porous plastic, containing an equal volume of immobilized TPCK–trypsin. The samples were incubated for 1 h at 4 °C in the dark and then centrifuged for 30 s at 1000g to separate the phytochrome and its trypsin-digested peptides from the immobilized trypsin. These samples were either diluted with electrophoresis sample buffer for SDS–PAGE or used without further treatment for high-performance liquid chromatography (HPLC). No further

digestion of the samples occurred after the centrifugation step. The immobilized trypsin was washed and reused unless the applied sample contained a cross-linking reagent before digestion.

Size-Exclusion Chromatography. A Beckman (Arlington, IL) System 342 HPLC equipped with a Model 165 detector (dual wavelength, scanning capability) was used for all the analytical chromatography. A Reodyne filter (Deerfield, IL) and a TSK guard column were always used in-line prior to the analytical column. The columns, TSK 3000SW, TSK 4000SW (30 and 60 cm), TSK 5000PW, and the TSK guard (SW and PW), were from Toya Soda (Tokyo, Japan). Phytochrome samples (SAR = 0.85) were always centrifuged for 3 min in a Beckman microfuge prior to injection and were monitored simultaneously at 280 and 656 nm during the chromatography. The following proteins were used to calibrate the columns: thyroglobulin, apoferritin, β-amylase from sweet potato, alcohol dehydrogenase from yeast, phosphorylase *b*, bovine serum albumin, and carbonic anhydrase.

SDS–PAGE and Immunoblotting. SDS–PAGE was performed under white light according to the method of Laemmli (1970). Acrylamide gels (0.75 mm thick) were blotted onto nitrocellulose sheets (HAHY, Millipore, Bedford, MA) at 150 mA for 3 h. The transfer buffer contained 25 mM Tris, 200 mM glycine, and 20% methanol. Blots were incubated overnight at 4 °C in 100 mM Tris, pH 7.4, 150 mM NaCl, 3% Carnation evaporated milk (Carnation Corp., Los Angeles, CA), and 0.02% NaN_3 (milk buffer; Johnson et al., 1984). All washes and Ig dilutions were made with the milk buffer. Primary incubations with monoclonal Ig were 3 h while secondary incubations with anti-mouse Ig (prepared in rabbit), tertiary incubations with anti-rabbit Ig (prepared in goat) conjugated with alkaline phosphatase, and the intermediate wash steps were each 1 h. The primary incubations with polyclonal Ig were 2 h. The blots were stained by overlaying them with 2% agar gels containing 1 mM 5-bromo-4-chloroindole phosphate and 0.75 M Tris, pH 9.8 (Knecht & Dimond, 1984). Coomassie blue conjugated standards were used to calibrate all blots. The apparent molecular masses of the dye-conjugated standards were determined by direct comparison on blots with nonconjugated standards visualized with Ponceau stain (Pratt, 1984). The apparent molecular masses of Coomassie-conjugated BSA and ovalbumin were determined to be 69 and 46 kDa, respectively. The other Coomassie-conjugated standards, myosin H chain, phosphorylase *b*, and α-chymotrypsinogen, comigrated with their respective nonconjugated standards. Phytochrome samples in SDS electrophoresis buffer were heated to 60 °C in a water bath for 5–10 min before they were subjected to SDS–PAGE. We have found that boiling phytochrome in SDS buffer causes cleavage at aspartyl–prolyl peptide bonds, resulting in major peptides with molecular masses of 112, 64, 60, and 47 kDa. These "boiling" peptides comigrate with peptides produced by the formic acid digestion method of Sonderegger et al. (1982) and have the expected molecular masses based on aspartyl–prolyl bond positions determined from a phytochrome cDNA clone (Hershey et al., 1985). Rittenhouse and Marcus (1984) have recently demonstrated that boiling can induce cleavage at aspartyl–prolyl bonds.

Results

Sedimentation Equilibrium Centrifugation. Highly pure, 124-kDa phytochrome (SAR = 1.11) was centrifuged at low speed (4000 rpm) for 48 h (equilibrium), and the distribution of protein in the cell was determined with scanning optics. A plot of the natural log of the concentration vs. the square of the distance from the rotor center is a straight line, indicating that there is a homogeneous population with a single molecular mass of 253 kDa (solid line, Figure 1). The phytochrome used in these experiments chromatographed as a single peak on a TSK 4000SW column both before and after the centrifugation. Also, immunoblot analysis revealed that less than 5% of the phytochrome was degraded during the run.

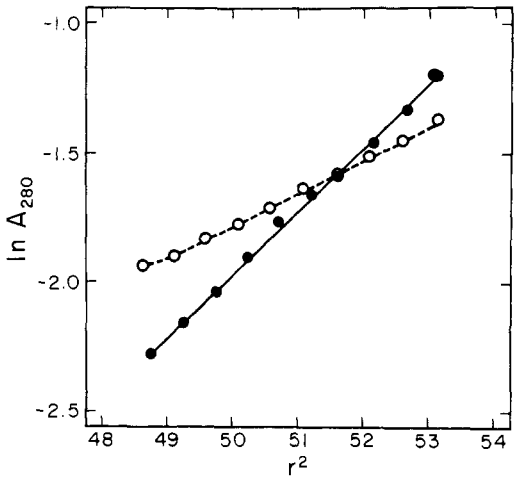

FIGURE 1: Sedimentation equilibrium centrifugation of phytochrome. Phytochrome (SAR = 1.11) was centrifuged as described under Materials and Methods in the presence (○) or absence (●) of 1 M NaCl. Slopes of the lines were determined by linear regression. The absorbance data were taken over the whole length of the centrifugation cell.

When 1 M NaCl was included during centrifugation, the plot appeared slightly curvilinear, representing an average molecular mass of approximately 155 kDa (dashed line, Figure 1). Higher speed centrifugation (6000 rpm) with 1 M NaCl gave better resolution. In this case, the lower limiting slope indicated a molecular mass of 147 kDa, and the average molecular mass across the cell was 196 kDa (data not shown).

Cross-Linking with Dimethyl Suberimidate and Glutaraldehyde. Phytochrome purified by the method of Vierstra et al. (1983a) apparently contains a small proportion of covalently cross-linked dimers that migrate at approximately 265 and 245 kDa on SDS–PAGE (Figure 2, lane a). These high molecular mass bands are recognized by all of our classes of monoclonal antibodies directed against phytochrome (Daniels & Quail, 1984) and are not dissociated by extensive boiling or high levels of SDS or β-mercaptoethanol (data not shown). They possibly result from cross-linking by quinones in the crude extract (W. O. Smith, personal communication; Cha et al., 1983) and will be designated "control cross-linked" for convenience. If phytochrome is cross-linked with dimethyl suberimidate (DMS), a new band at 225 kDa is observed, as well as an increase in intensity for the control-cross-linked bands (Figure 2, lanes b–e). Dimethyl pimelimidate (DMP) and dimethyl adipimidate (DMA) yield similar results (data not shown). Bands corresponding to presumptive trimers and tetramers are rarely observed. The function of molecular masses for cross-linked products above 150 kDa is not strictly linear with mobility in SDS–PAGE. This result is not unexpected since deviation from an open chain has been shown to be serious with cross-linking and since cross-link span and position can alter SDS binding, SDS-induced unfolding, and stable denatured conformation (Steele & Nielsen, 1978). Complete cross-linking did not occur even at pH 9.2 with any of the imido esters.

Glutaraldehyde effectively cross-links phytochrome to a heterogeneous population with a mean of approximately 250

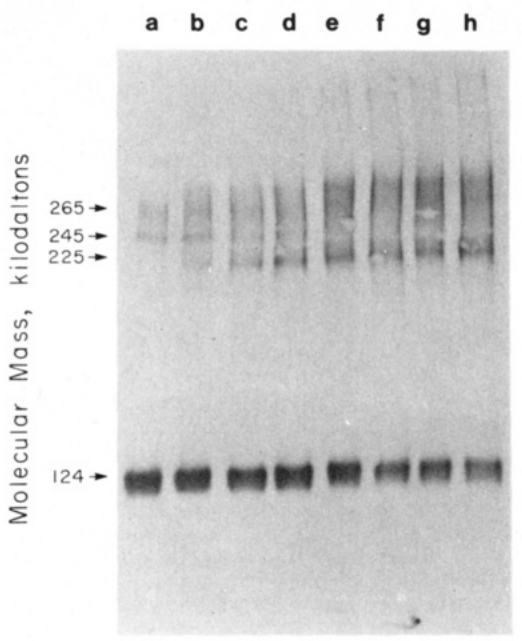

FIGURE 2: Cross-linking phytochrome with DMS. Phytochrome was cross-linked with DMS as described under Materials and Methods. The controls and DMS-cross-linked phytochrome samples were subjected to SDS–PAGE (5% acrylamide) and immunoblot analysis. The blot was probed with polyclonal Ig directed against 124-kDa phytochrome. The mass ratio of DMS to phytochrome in each reaction was as follows: lane a, zero DMS; lane b, 1; lane c, 2; lane d, 5; lane e, 10; lane f, 20; lane g, 5 added twice in 1 h; lane h, 5 added 3 times in 1 h.

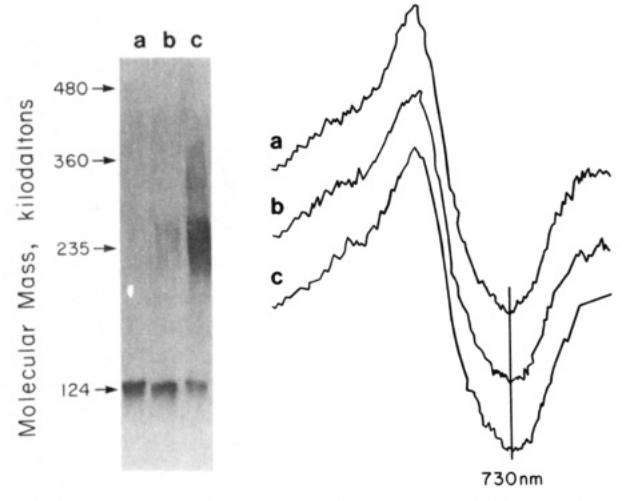

FIGURE 3: Cross-linking phytochrome with glutaraldehyde. Phytochrome was cross-linked with glutaraldehyde as described under Materials and Methods. Samples were subjected to SDS–PAGE (5% acrylamide) and immunoblot analysis. The blot was probed with polyclonal Ig directed against 124-kDa phytochrome. (a) Zero glutaraldehyde; (b) G/P ratio = 0.4 (pH 9.2); (c) G/P ratio = 2.2 (pH 9.2). The corresponding difference spectra are shown on the right. $\Delta\Delta A$ = 0.010.

kDa (Figure 3). At pH 9.2, the mass ratio of glutaraldehyde to phytochrome necessary for 50% cross-linking is approximately 1–2, whereas, at pH 7.8, it is 15–20, 10-fold higher than at the higher pH as expected from the cross-linking mechanism proposed by Habeeb and Hiramoto (1968). The difference spectra for phytochrome cross-linked to 50% are unaltered (Figure 3). Only dimers were formed at pH 7.8 over a concentration range of phytochrome of 6–66 μg/mL in the presence or absence of 150 mM NaCl and with constant or increasing mass ratios of glutaraldehyde to phytochrome (data not shown).

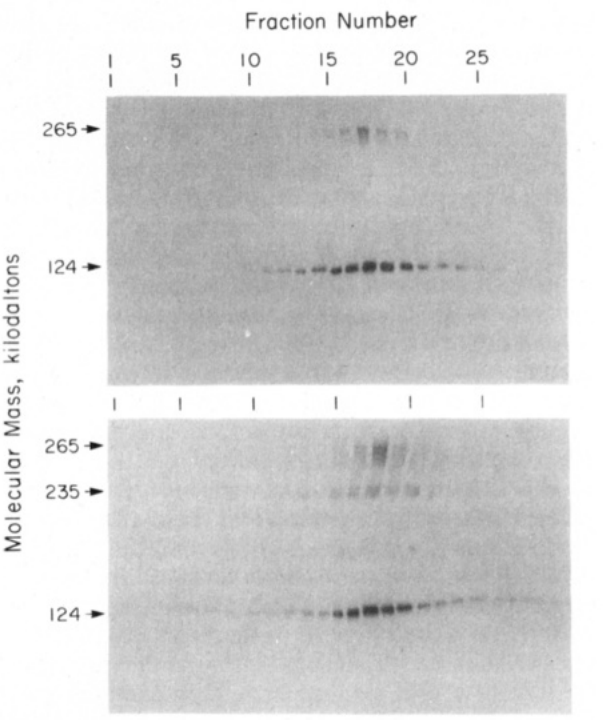

FIGURE 4: SEC of DMS-cross-linked phytochrome. Phytochrome either un-cross-linked (top panel) or partially cross-linked with DMS (bottom panel) was fractionated on a TSK 3000SW column under nondenaturing conditions (ordinate) and then subjected to SDS–PAGE (abscissa) and immunoblot analysis. Blots were probed with polyclonal Ig directed against 124-kDa phytochrome.

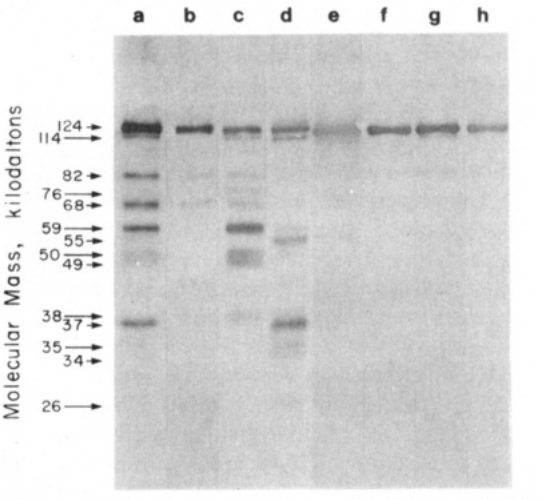

FIGURE 5: Trypsin digestion of phytochrome. Phytochrome (124 kDa, Pfr) was digested with trypsin as described under Materials and Methods. Digested (lanes a–d) and undigested (lanes e–h) samples were subjected to SDS–PAGE (10% acrylamide) and immunoblot analysis by probing with polyclonal Ig (lanes a and e), type 1 monoclonal Ig (lanes b and f), type 2 monoclonal Ig (lanes c and g), or type 3 monoclonal Ig (lanes d and h).

Size-Exclusion Chromatography of DMS-Cross-Linked Phytochrome. Both control-cross-linked (Figure 4, top panel) and DMS-cross-linked (Figure 4, bottom panel) phytochromes comigrate with un-cross-linked phytochrome under nondenaturing conditions in SEC with an approximate molecular mass of 300 kDa. These observations indicate that (1) the un-cross-linked phytochrome migrating at 300 kDa behaves as a dimer and that (2) DMS, at least at the ratio used here, does not cause gross conformational changes in the molecule. The same results were obtained with glutaraldehyde-cross-linked phytochrome (data not shown).

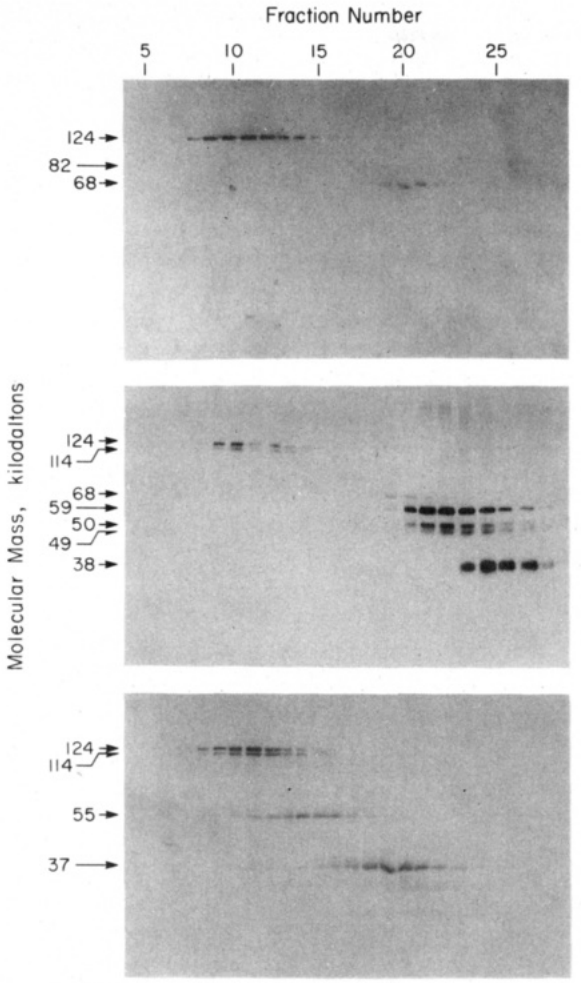

FIGURE 6: SEC of trypsin-digested phytochrome probed with monoclonal Ig's. Phytochrome was digested with trypsin as described under Materials and Methods and then fractionated on a TSK 3000SW column under nondenaturing conditions. The eluent was 100 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, and 1 mM EDTA. The flow rate was 0.5 mL/min. Fractions were subjected to SDS–PAGE (10% acrylamide) and immunoblot analysis. Immunoblots were probed with type 1 monoclonal Ig (top panel), type 2 monoclonal Ig (center panel), and type 3 monoclonal Ig (bottom panel).

Size-Exclusion Chromatography of Trypsin-Digested Phytochrome. Phytochrome, as Pfr, was digested with trypsin for 1 h at 4 °C. The major products are 82, 68, 59, 55, 38, and 37 kDa (Figure 5a). The origin of each peptide was determined by probing the immunoblots with monoclonal Ig's which recognize spatially defined epitopes on the phytochrome polypeptide (Daniels & Quail, 1984). The 124-, 82-, and 68-kDa peptides are recognized by a type 1 Ig (Figure 5b), indicating that these peptides contain an intact amino terminus (Daniels & Quail, 1984). Type 2 Ig's, which recognize epitopes in the chromophore-bearing amino half of the molecule, bind to all the peptides except the ones with molecular masses of 55 and 37 kDa (Figure 5c). Type 3 Ig's, which bind to an epitope located between 27 and 36 kDa from the carboxyl terminus (Daniels & Quail, 1984), recognize the 37-, 55-, 114-, and 124-kDa phytochromes, indicating that these peptides contain the carboxyl terminus (Figure 5d). Undigested controls are shown in lanes e–h.

Samples of phytochrome were digested with trypsin, fractionated under nondenaturing conditions by SEC, and subjected to SDS–PAGE/immunoblot analysis utilizing monoclonal antibodies (Figure 6). The position of the peptides along the abscissa axis is a function of the native size, whereas

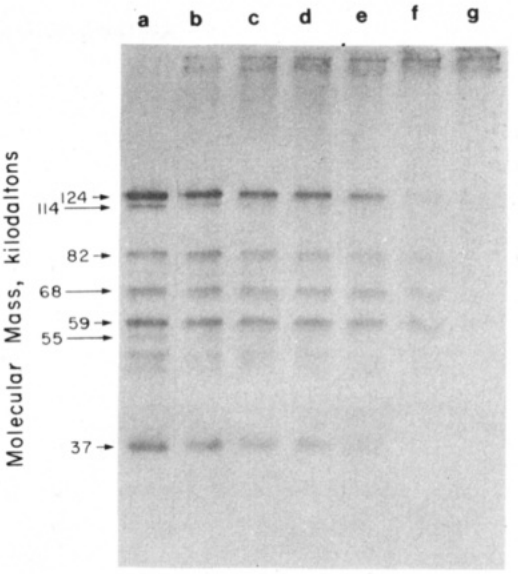

FIGURE 7: Glutaraldehyde cross-linking of trypsin-digested phytochrome. Phytochrome was digested with trypsin as described under Materials and Methods, cross-linked at the indicated glutaraldehyde to phytochrome mass ratios (pH 7.8), and subjected to immunoblot analysis: lane a, zero glutaraldehyde; lane b, 4; lane c, 8; lane d, 12; lane e, 16; lane f, 32; lane g, 80. The blot was probed with polyclonal Ig directed against 124-kDa phytochrome.

the position along the ordinate axis is the SDS-denatured size. Undigested phytochrome and the 114-kDa fragment migrate as 300-kDa entities (Figure 6). The 82-, 68-, and 59-kDa amino-terminal fragments, all missing at least 42 kDa from the carboxyl terminus, migrate as globular proteins in SEC with molecular masses approximately the same as determined by SDS–PAGE (Figure 6, top and middle panels). The 55- and 37-kDa fragments, which are derived from the carboxyl terminus, migrate as much larger peptides of 160 and 74 kDa, respectively, in SEC (Figure 6, bottom panel). This result indicates that these peptides either are aggregated or are monomeric with a shape that deviates greatly from globular. All peptides carrying the type 2 epitope also contain the chromophore as monitored spectrophotometrically (data not shown).

Glutaraldehyde Cross-Linking of Tryptic Peptides from Phytochrome. Phytochrome digested with trypsin was treated with glutaraldehyde at the indicated glutaraldehyde to phytochrome (G/P) mass ratios (Figure 7). The decreasing intensities of the tryptic peptide bands observed with increasing glutaraldehyde are taken to indicate cross-linking, despite the absence of detectable levels of new bands representing the cross-linked products. The alternative, that band disappearance simply represents destruction of epitope reactivity by chemical modification, is unlikely given that the relative band intensities of the 82-, 68- and 59-kDa peptides persist at higher glutaraldehyde levels than for the 124-kDa polypeptide from which they are derived (Figure 7). The lack of detectable cross-linked products may result from a combination of the relatively low amounts of each individual peptide and the polydisperse behavior of the cross-linked products formed by glutaraldehyde (Figure 3). Complete cross-linking of all peptides occurs at high enough ratios as indicated by their disappearance, but some of the fragments appear to be more resistant to cross-linking than others (Figure 7).

The amount of each peptide present in each lane was determined by scanning photographic negatives of immunoblots probed with both polyclonal and monoclonal Ig's. The amount remaining at each glutaraldehyde level is expressed as a

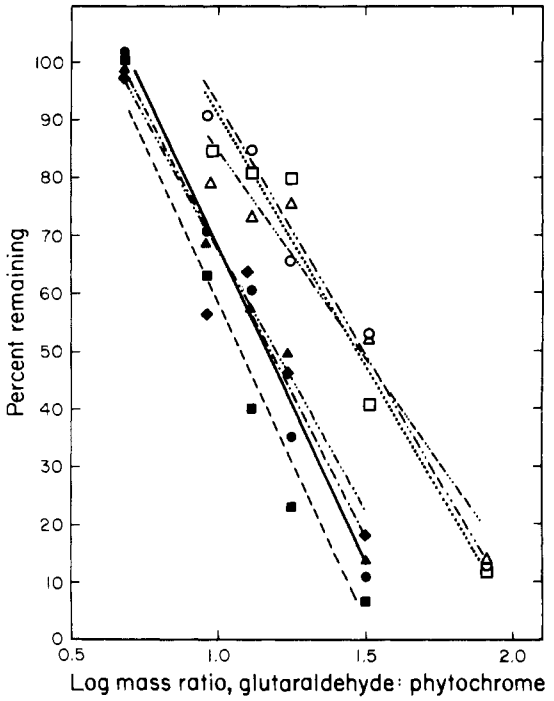

FIGURE 8: Quantitative analysis of phytochrome tryptic peptides cross-linked with glutaraldehyde. The amount of each trypsin-derived peptide remaining as a function of increasing G/P ratio was determined by scanning photographic negatives of immunoblots probed with polyclonal or monoclonal Ig. The percent remaining value is expressed as a percent of the zero glutaraldehyde control (Figure 7, lane a) at each of the indicated G/P ratios. The type of Ig used on the blot from which the data are derived and the correlation coefficient of the linear regression are indicated respectively in parentheses: 124-kDa band (polyclonal, 0.98) [— (●)]; 82-kDa band (polyclonal, 0.97) [- - ·· - - (○)]; 68-kDa band (polyclonal, 0.94) [··· (□)]; 59-kDa band (polyclonal, 0.92) [- - ··· - - (△)]; 55-kDa band (type 3 monoclonal, 0.98) [- - - (▲)]; 37-kDa band (type 3 monoclonal, 0.91) [- ·· - (♦)]; 37-kDa band (polyclonal, 0.91) [- - - (■)]. This experiment was repeated 3 times with the same results.

percentage of the zero glutaraldehyde control value for each individual peptide (Figure 8). Two distinct classes of peptide are apparent: one which is cross-linked at low glutaraldehyde to phytochrome ratios (designated the "early" class) and another class which is cross-linked at G/P ratios 2–3 times higher (designated the "late" class). All the readily cross-linked peptides in the early class contain or are derived from the carboxyl-terminal 42-kDa fragment, whereas the loss of this fragment by proteolytic cleavage renders these peptides more resistant to glutaraldehyde cross-linking.

To determine whether the apparent aggregation of the 55- and 37-kDa, carboxyl-derived peptides observed in SEC and in the glutaraldehyde cross-linking experiments is induced artifactually by trypsin digestion, phytochrome was first cross-linked and then digested with trypsin before being subjected to SDS–PAGE/immunoblot analysis. Under these conditions, the 55- and 37-kDa peptides are once again readily cross-linked, falling into the early class together with the parent 124-kDa species (data not shown). Likewise, the 68-kDa amino-terminal peptide is once more less readily cross-linked, again exhibiting late class behavior. The 82- and 59-kDa peptides, however, fall into the early class under these conditions. The reason for this unexpected result is currently unknown.

Size-Exclusion Chromatography. The 124-kDa phytochrome was chromatographed under a variety of conditions to determine the type of interaction involved in dimerization (Table I). The molecule behaves as a 350-kDa protein in Tris

Table I: Size-Exclusion Chromatography of Phytochrome

column	buffer	app mol mass (kDa)	obsd difference, Pfr – Pr retention
TSK 4000SW	Tris, pH 7.2[a]	350	yes
TSK 3000SW	Tris, pH 7.2[a]	350	yes
TSK 3000SW	Tris, pH 6.2[b]	300	no
TSK 3000SW	NaP_i, pH 7.2[c]	300	no
TSK 3000SW	NaP_i, pH 7.2, 10 mM DTT	300	no
TSK 3000SW	NaP_i, pH 7.2, 1 M NaCl	170, V_0	no
TSK 5000PW	Tris, pH 9.2[b]	350	yes
TSK 5000PW	Tris, pH 7.8[b]	350	yes
TSK 5000PW	Tris, pH 6.4[b]	180	no

[a] 100 mM Tris, 150 mM NaCl, and 1 mM EDTA. [b] 100 mM Tris, 200 mM NaCl, and 1 mM EDTA. [c] 100 mM sodium phosphate, 150 mM NaCl, and 1 mM EDTA. Flow rate = 0.5 mL/min.

buffers on both silica-based (TSK 3000SW, TSK 4000SW) and polymer-based (TSK 5000PW) supports. The previously described differences in retention between Pr and Pfr (Lagarias & Mercurio, 1984; Smith & Cyr, 1983) were also observed here, but only when Tris buffered at pH 7.2–9.2 was used. Phytochrome in sodium phosphate buffer migrated as a protein with a molecular mass of 300 kDa, and there was no difference in retention between Pfr and Pr.

The inclusion of 10 mM DTT did not alter the position or shape of the phytochrome peak, confirming that disulfide bridges are not exclusively involved in maintaining phytochrome dimers (Hunt & Pratt, 1980). However, 1 M NaCl moves the position of the phytochrome peak to correspond to a molecular mass of 170 kDa. In Tris buffered at pH 6.4, phytochrome migrated as a sharp peak with a molecular mass of 180 kDa on the TSK 5000PW column but not on the TSK 3000SW column, indicating that the increased retention on the polymer-based PW column is probably due to an interaction with the matrix.

Discussion

Evidence for a Dimeric Structure. Understanding the quaternary structure of phytochrome is an essential part of understanding the molecular mechanism of its action. Previous results were interpreted to indicate that phytochrome is dimeric [reviewed by Briggs & Rice (1972) and Hunt & Pratt (1980)]. However, two major problems with these results require that this interpretation be qualified. In all previous studies, either the molecule examined was most likely partially degraded and/or the methods used to estimate the molecular mass gave results which were dependent upon the molecular shape. We have addressed these limitations by analyzing undegraded, 124-kDa phytochrome using a method that gives shape-independent results.

We interpret data from three independent sets of experiments to indicate that undegraded, 124-kDa phytochrome from etiolated oats is a dimer in solution. The direct evidence comes from sedimentation equilibrium centrifugation since data obtained with this method are independent of shape. The low g force used in our study has the advantage that the concentration gradient across the centrifuge cell is shallow, thus minimizing the possibility of artifactual self-association due to high protein concentrations or steep gradients (Van Holde, 1975). The disadvantage is the long times needed to reach equilibrium with a full cell, and therefore, the possibility of degradation is increased. To address this problem, we used phytochrome with high purity (SAR = 1.11), a short column, since the time to equilibrium is proportional to the square of the column height (Bowen & Rowe, 1970), and low temper-

ature, and we monitored directly for degradation and irreversible aggregation after the run by SDS–PAGE and SEC, respectively. Over a concentration range of 0.1–0.35 mg/mL, phytochrome, within the limits of detection, is exclusively a dimer of 253 kDa. In high salt, the average molecular mass is greatly reduced, presumably as a result of partial subunit dissociation (Klotz et al., 1975).

In cross-linking experiments utilizing a variety of reagents, the major cross-linked product detected on SDS–PAGE always migrates with the apparent mass expected of a dimer in the range of protein concentrations tested (6–66 μg/mL). This result also indicates that at low concentrations, such as in vivo (approximately 100 μg/mL cytoplasm), the predominant form of phytochrome is a dimer. Four conclusions can be drawn from the observation that cross-linked phytochrome comigrates with un-cross-linked phytochrome in SEC under nondenaturing conditions. First, the quaternary structure of cross-linked phytochrome is similar to that of the un-cross-linked molecule. Second, the apparent large volume occupied by phytochrome in SEC is not due to an extremely elongated molecule consisting of a single polypeptide chain nor to aggregation of an order higher than 2. Third, the range of cross-linked phytochrome bands observed in SDS–PAGE is probably due to differences in their denatured conformations, not differences in their masses. Fourth, the conditions used in the cross-linking reactions are sufficiently mild that gross conformational changes do not occur. This latter conclusion is also confirmed by the unchanged spectra of a phytochrome sample where at least 50% is colvalently cross-linked. This observation is consistent with the results of Roux and Hillman (1969), who have shown that at a ratio of glutaraldehyde to Pfr 100 times higher than that used here to achieve 50% cross-linkage, the $\Delta\Delta A$ was only reduced 50% compared to untreated controls.

Localization of the Dimerization Region. Early data [see references in Briggs & Rice (1972)] indicated that the 60-kDa, chromophore-bearing half of degraded phytochrome ("small phytochrome"), when released from the intact molecule, is a globular monomer in SEC. From this result and the later report that 118/114-kDa phytochrome is a dimer (Hunt & Pratt, 1980), it might have been tempting to deduce that the dimerization site must reside in the non-chromophore-containing half of the polypeptide. However, because this 60-kDa degradation product lacks the structurally important 10-kDa, amino-terminal peptide segment (Daniels & Quail, 1984; Jones et al., 1985), it could not be excluded that the amino-terminal domain of phytochrome is directly involved in dimerization via contact sites in this 10-kDa segment. The data presented here eliminate that possibility by showing that all peptides of 82 kDa or smaller with intact amino termini behave as globular monomers.

Direct support for the conclusion that the dimerization site lies within the carboxyl-terminal domain is provided by the following observations. (a) Trypsin-generated peptides which contain the carboxyl-terminal 42 kDa behave as larger molecules, 2–3 times their monomeric molecular mass, under nondenaturing conditions. (b) Peptides containing this 42-kDa domain become cross-linked with glutaraldehyde at lower concentrations than those without this region. Thus, the data obtained here with tryptic digests of purified phytochrome confirm and extend those derived from proteolysis of the photoreceptor in crude preparations by endogenous proteases (Jones et al., 1982; Vierstra et al., 1983). The present evidence narrows the region within which the dimerization contact site(s) is (are) likely to be located from 55 to 42 kDa from the carboxyl terminus. The evidence indicating that this carboxyl domain contains the dimerization site(s) is summarized in Figure 9.

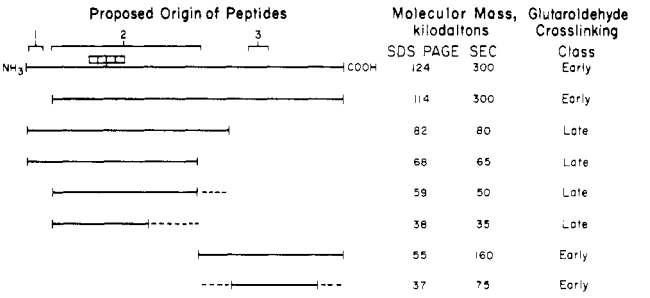

FIGURE 9: Peptide map and characteristics of trypsin-generated peptides from phytochrome. Peptides (solid lines) resulting from trypsin digestion of 124-kDa phytochrome are shown on the left at their proposed origin. Broken lines represent the extent of a region from which the peptides are derived and indicate uncertainty as to the exact location of the fragment within this region. The regions containing the epitope location of monoclonal antibodies designated types 1, 2, and 3 (Daniels & Quail, 1984) are shown above the 124-kDa molecule. The molecular mass determined by SDS–PAGE and SEC is shown to the right of each peptide. Also to the right is indicated the propensity to be cross-linked with glutaraldehyde. Early indicates that these peptides are cross-linked at relatively low G/P ratios; late, at higher G/P ratios.

It appears unlikely that the large molecular mass of the cleaved, carboxyl peptides observed in SEC under nondenaturing conditions is due either to higher order aggregation (e.g., trimers) or to a highly elongated monomer configuration. Trimers would be expected to cross-link at lower G/P ratios than for the intact dimer (early class). Conversely, monomers would be expected to cross-link at higher G/P ratios than for the intact dimer, i.e., at the same ratios as for the globular, amino-terminal peptides (late class). Neither of those alternatives is observed. This result suggests that the trypsin-generated, carboxyl peptides are indeed dimers. Furthermore, since the domains from which these fragments are derived are preferentially cross-linked in the intact molecule, it is also unlikely that the dimerization of these peptides is induced by trypsin cleavage.

Molecular Shape. Nondenaturing, size-exclusion chromatography of 124-kDa phytochrome and of the trypsin-generated peptides has provided information on the shape of the native molecule as well as that of the monomer and its domains. Phytochrome, in SEC under nondenaturing conditions, has an apparent molecular mass of 300–350 kDa (Table I; Lagarias & Mercurio, 1985). The Stokes radius determined from these data is 56 Å, which is similar to other published values for oat phytochrome where analytical chromatography was used to make the estimation (56 Å, Lagarias & Mercurio, 1985; 61 Å; Litts, 1980). However, Sarkar et al. (1984) report a much larger Stokes radius (81 Å), calculated from the diffusional coefficient determined by a quasi-elastic light-scattering technique. The reason for this discrepancy is not known.

The frictional ratio (f/f_0), a coefficient indicating the degree of deviation of the molecular shape from a sphere, is estimated here to be 1.37 for the undegraded dimer, indicating that native phytochrome has an elongated axis. The apparent monomers observed in high-salt conditions also behave as elongated molecules in SEC (170 kDa, $f/f_0 = 1.34$). Conceivably, salt could induce this elongated conformation by denaturation, but preliminary evidence that $\Delta\Delta A$ and the absorbance peaks are unchanged in high salt (A. M. Jones and P. H. Quail, unpublished experiments) lessens this possibility. These data, therefore, suggest that phytochrome is composed of two elongated monomers. As indicated above, the amino-terminal

domain of each monomer appears to be globular in SEC. Previous data (Jones et al., 1985) suggest that this amino-terminal domain is probably globular in the undigested molecule as well, since all spectral parameters tested remained the same after this domain was released from the intact molecule. The trypsin-released, still-associated carboxyl domains behave as an elongated entity in SEC, since the f/f_0 value is large (1.44) for the 110-kDa dimer (two 55-kDa monomers). However, we are unable to determine from present data whether the elongated character of this dimerized carboxyl region is due to two 55-kDa domains that are individually globular or elongated. In favor of the latter possibility is the observation by ourselves and others (Jones et al., 1985; Lagarias & Mercurio, 1985) that the carboxyl domain is more accessible to protease digestion than the amino domain, suggesting that it may have an extended rather than a compact conformation.

Lagarias and Mercurio (1985) have reported differences in retention times for Pfr and Pr in Tris buffer. We confirm the increased retention of Pr using Tris buffers on the TSK 3000SW column. We do not, however, observe these differences with NaP_i buffers. The differential retention of Pfr and Pr could be due to either different molecular volumes or different affinities of the two molecular forms for the column support. Since we find the same difference in retention on a completely different support (TSK 5000PW vs. TSK 3000SW), the differences are most likely due to volume differences as Lagarias and Mercurio (1985) have previously suggested. The observed increased retention of phytochrome on the TSK 5000PW column with Tris buffered at pH 6.4 vs. 7.2, however, is due to a pH-dependent interaction with the column matrix since the elution volume at pH 6.4 is the same as at pH 7.2 with a different support (TSK 3000SW).

Nature of the Forces Involved in Dimerization. The presence of dithiothreitol does not alter the elution position or peak shape of the phytochrome dimer upon SEC, indicating that disulfide bonds are not exclusively involved in maintaining the association between the two monomers. This result is in agreement with an earlier conclusion by Hunt and Pratt (1980) on the involvement of disulfide bridging in partially degraded phytochrome. These workers found that the migration of 118/114-kDa phytochrome in nondenaturing gel electrophoresis was unaffected by 0.1 mM β-mercaptoethanol, a 100-fold less concentration of reductant than that used in the present study. In contrast, data from analytical centrifugation and SEC experiments show that a high salt concentration partially dissociates the dimers, providing evidence that the contact site may be ionic in nature.

In summary, our data indicate that phytochrome is a dimer in solution at concentrations well above and below in vivo levels. The phytochrome dimer is elongated and appears to be composed of two elongated monomers each with a globular amino-terminal domain. The contact site for the dimer is located within 42 kDa from the carboxyl terminus and may be partially ionic in nature.

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Biosynthesis of an Asparagine-Linked Oligosaccharide-Containing Calcitonin by a Rat Medullary Thyroid Carcinoma Cell Line†

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ABSTRACT: Calcitonin contains an amino acid sequence that provides a potential site for glycosylation of the peptide at the asparagine at position 3. Preliminary evidence has suggested that there are glycosylated forms of calcitonin and its precursor, procalcitonin. The CA-77 rat medullary thyroid carcinoma cell line, recently developed to study calcitonin biosynthesis, was used to demonstrate the synthesis of glycosylated forms of this hormone by intact cells. Cultures were incubated in medium containing either [^{3}H]mannose or [^{35}S]methionine. Two species incorporating both labels were specifically immunoprecipitated when cell extracts were treated with calcitonin antibodies. Gel filtration chromatography in 6 M guanidine hydrochloride indicated that one peptide had a molecular weight of 5500, approximately 2000 daltons larger than calcitonin, while the second peptide had a molecular weight of 14 400, the approximate size of procalcitonin. Treatment of the [^{3}H]mannose-labeled cell extract with endo-β-*N*-acetylglucosaminidase H before immunoprecipitation removed the labeled sugar from the calcitonin species. Microsequence analysis of the radiolabeled immunoreactive 5500-dalton calcitonin species showed methionine at cycle 8 and mannose at cycle 3, suggesting that this peptide is calcitonin containing an N-linked oligosaccharide at Asn-3. These results suggest that in this cell line a minor but significant biosynthetic pathway exists for the production of glycosylated calcitonin from glycosylated procalcitonin.

The 32 amino acid sequence of the peptide hormone calcitonin contains an N-linked glycosylation site (Raulais et al., 1976; Struck et al., 1978). This sequence, Asn-Leu-Ser, at residues 3–5 (Raulais et al., 1976), is a necessary but not compulsory condition for the transfer of an oligosaccharide consisting of glucose, mannose, and *N*-acetylglucosamine from a dolichol phosphate donor to the amide nitrogen of the asparagine (Waechter & Lennarz, 1976; Wagh & Bahl, 1981). In vivo such sites are often not recognized, or only a fraction of the molecules is modified (Eipper et al., 1976; Nisbet et al., 1981). The major form of calcitonin isolated from various mammalian and nonmammalian sources is not glycosylated (Guttman, 1981). Thus, it would appear that the signal was not recognized in vivo. Recently, several laboratories have reported that a fraction of the total immunoreactive calcitonin in extracts of calcitonin-producing tumors binds specifically to lectins, including concanavalin A (Dermody et al., 1981; O'Neil et al., 1981; Baylin et al., 1983). Furthermore, in the presence of microsomal membranes, cell-free translation of rat medullary thyroid carcinoma mRNA generates an N-linked glycosylated procalcitonin, the biosynthetic precursor of calcitonin (Jacobs et al., 1981), as expected from this type of experiment (Pless & Lennarz, 1977). In addition, synthetic calcitonin is a substrate for oligotransferases in the same in vitro system which will glycosylate procalcitonin (Jacobs et al., 1985).

Birnbaum et al. (1985) have described the major pathway for the biosynthesis of calcitonin in the dexamethasone-treated CA-77 cell line. Procalcitonin is proteolytically processed to a 6500-dalton biosynthetic intermediate, which is subsequently cleaved to the amidated, 32 amino acid calcitonin. We have observed (Birnbaum et al., 1984a) multiple forms of procalcitonin by reversed-phase high-performance liquid chromatography (HPLC)[1] fractionation of immunoprecipitates of radiolabeled CA-77 cell extracts or microsomal membrane

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[1] Abbreviations: HPLC, high-performance liquid chromatography; Endo H, endo-β-*N*-acetylglucosaminidase H; 3.4K etc., molecular weights ($\times 10^{-3}$) estimated by gel filtration chromatography; ACTH, adrenocorticotropin.

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