Surface Topography of Phytochrome A Deduced from Specific Chemical Modification with Iodoacetamide[†,‡]

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ABSTRACT: Phytochromes are a photoreversible photochromic light switch for photomorphogenesis in plants. The molecular structure and functional mechanism of phytochromes are not fully understood. On the basis of complete mapping of total tryptic digest of the iodoacetamide-modified oat phytochrome A (phyA), the molecular surface topography of phyA was probed by specific chemical modification of cysteine residues with [^{14}C]iodoacetamide. Under native conditions, only two cysteines (Cys-158 and Cys-311) of eleven half-cystines of the N-terminal chromophore binding domain were modified to a significant extent. In the C-terminal domain, six cysteine residues (Cys-715, Cys-774, Cys-809, Cys-869, Cys-961, Cys-995) were readily accessible to iodoacetamide. Among the reactive cysteine residues, only cysteine-311 displayed reactivity that was dependent on the photochromic form (Pr ⇆ Pfr) of the photoreceptor. Surprisingly, the modification of Cys-311 in the vicinity of the chromophore attachment site (Cys-321) did not have any detectable effect on spectral properties of phyA. Most of the cysteines of the N-terminal domain (Cys-83, Cys-175, Cys-291, Cys-370, Cys-386, Cys-445, Cys-506) are deeply buried in the core of the chromophore binding domain, as they can be modified only after denaturation of the chromoprotein. In the C-terminal domain, modification of only one cysteine residue (Cys-939) required protein denaturation. Since all 22 half-cystines can be modified with iodoacetamide without reduction of the chromoprotein, it follows that oat phyA does not have any disulfide bonds. We found that Cys-311, Cys-774, Cys-961, and Cys-995 could be easily partially oxidized under the conditions used for phytochrome isolation. The surface topography/conformation of oat phyA and its role in protein−protein recognition in phytochrome-mediated signal transduction are discussed in terms of the relative reactivity of cysteine residues.

Phytochromes are a light switch which mediates many biochemical and physiological processes in higher plants in response to red/far-red light (*1*−*4*). Phytochromes are cytosolically localized proteins composed of two subunits, each approximately 124 kDa. The chromophore, phytochromobilin, is covalently linked to cysteine-321 in oat phytochrome A (phyA)[1] (*5*). Phytochromes exist in two photochromic forms, red-light-absorbing Pr and far-red-light-absorbing Pfr forms. The latter is considered to be a signal-mediating, "switch on" form.

Phytochromes are the best characterized plant photoreceptors. However, the pathway of biochemical events from light absorption to biological response remains obscure. Although phyA and phyB, and possibly other members of the phytochrome family, have different physiological functions, there is evidence that the biochemical mechanisms for downstream signaling may be very similar, if not identical (*4*, *6*).

PhyA represents 98% of phytochrome pool in etiolated tissue (*7*). The protein can be described in terms of two domains: a globular N-terminal chromophore domain and a more extended C-terminal domain, which is responsible for dimerization of the chromoprotein (*8*−*14*). The N-terminal domain, apart from being the determinant for photoperception, contains motifs which are necessary for biological activity (*15*−*17*). Two discrete regions at both ends of the C-terminal domain are also required for transduction of the light signal (*4*, *15*, *16*, *18*). The present study was undertaken to characterize the protein surfaces of both domains for possible protein−protein interactions. For this purpose, we probed the surface cysteine residues in phyA.

The nucleotide-derived sequence of oat phyA contains 23 half-cystine residues (*19*), with Cys-321 for chromophore linkage. Previous data indicate that oat phyA has two disulfide bonds (*20*). A large number of half-cystines in oat

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[1] Abbreviations: CAM-phyA, carboxamidomethylated phytochrome A; CID, collision induced dissociation; ESIMS, electrospray ionization mass spectrometry; DTT, dithiothreitol; GdHCl, guanidine hydrochloride; phyA, phytochrome A; Pr and Pfr, red and far-red absorbing forms of phytochrome, respectively; SAR, specific absorbance ratio, A_{666}/A_{280}, with phytochrome in its red-absorbing form; TFA, trifluoroacetic acid.

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phyA provided us with a chemical probe to investigate the surface topography of phyA protein using SH-specific reagents. The half-cystine residues are located in the central parts of the N- and C-terminal domains of oat phyA, and little information is currently available on the conformation and topography of these regions. Previous studies have shown that sulfhydryl specific reagents can be used as probes for conformational changes in the native protein induced by Pr → Pfr phototransformation (*21*, *22*). Those studies, however, were performed at the level of large fragments of limited digests, that is, without sufficient "spatial" resolution. In this study, we have used a complete tryptic mapping of iodoacetamide-derivatized phyA to probe its conformation in both photochromic forms with [^{14}C]iodoacetamide. Oat phyA was selectively modified with [^{14}C]iodoacetamide under three different conditions: (1) To compare the reactivity of Cys residues in Pr and Pfr forms of phytochrome, the reaction was performed under native conditions; (2) To determine the potential sites for S−S bond formation, the radioactive label was attached to the cysteine residues which became accessible to the modification only after reduction; and (3) To identify "buried" cysteines, modification with [^{14}C]iodoacetamide in the presence of guanidine hydrochloride was carried out after prior blocking of accessible cysteines with 100 mM "cold" iodoacetamide. Results from these experiments were used to deduce the surface topography of phyA.

MATERIALS AND METHODS

Materials. Proteolytic enzymes, trypsin, pepsin, subtilisin (protease Type XXVII, Nagarse), thermolysin (protease type X), and proteinase Glu-C (type XVII), were purchased from Sigma Chemical Co (St. Louis, MO). Endoproteinase Asp-N was obtained from Boehringer Mannheim GmbH (Germany). Bio-Gel P-6 (fine) was purchased from Bio-Rad Laboratories (Oakland, CA). [1-^{14}C]Iodoacetamide (21.5 mCi/mmol) was obtained from NEN Products (Boston, MA). All other reagents were obtained from Sigma Chemical Co.

Phytochrome Preparations. Phytochrome A from etiolated *Avena sativa* L. Garry oat, with a specific absorbance ratio (SAR, $A_{666}/A_{280} = 1.03$ for Pr) was obtained essentially as described previously (*23*) by using an ammonium sulfate back-extraction procedure and gel filtration in 20 mM Tris-HCl buffer, pH 7.8, 1 mM dithiothreitol (DTT) on Toyo-Pearl HW-65.

Derivatization with Iodoacetamide. Peptide mapping was facilitated by derivatizing cysteinyl residues with iodoacetamide prior to tryptic digestion. All reactions with iodoacetamide were performed under argon in darkness. Any disulfide-bonded Cys residues that might have been present in the native phytochrome were reduced by incubating approximately 20 nmol (as monomer) of the protein with 50 mM DTT in 20 mM Tris-HCl buffer containing 2 mM EDTA, 6 M guanidine hydrochloride (GdHCl), pH 8.3, for 2 h at 35 °C. The solvent was changed to the same buffer with 0.1 mM DTT by gel-filtration chromatography (Bio-Gel P-6), and derivatization was performed by incubating the reduced protein with 100 mM iodoacetamide for 1 h at 20 °C.

Modification of Cys residues in native Pr and Pfr phyAs (20 nmol) was performed by their incubation in 20 mM Tris-HCl buffer (pH 7.8) containing 2 mM EDTA, 0.1 mM DTT at 0 °C with 0.8 mM [^{14}C]iodoacetamide (26000−31000 cpm/nmol) for 1 h. After removal of the unreacted label (Bio-Gel P-6 column), all unmodified Cys residues were derivatized with nonradioactive 100 mM iodoacetamide in 6 M GdHCl.

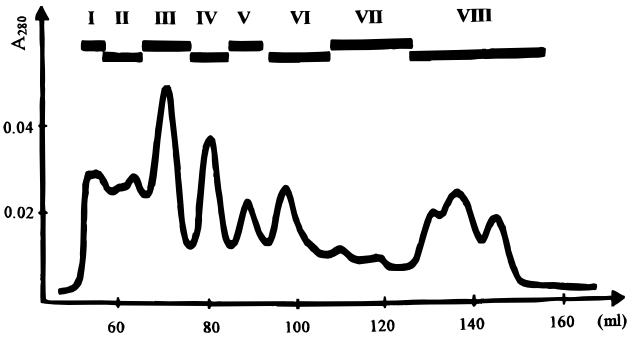

FIGURE 1: Gel-filtration chromatography of tryptic digest of oat phyA. PhyA modified with iodoacetamide as indicated under "Materials and Methods" was digested with trypsin for 5 h at 37 °C [trypsin−phytochrome ratio of 1:50 (w/w)]. The digest was applied to a Bio-Gel P-6 (1.5 × 95 cm) column equilibrated with 100 mM ammonium bicarbonate: flow rate, 10.4 mL/h; detection, at 280 nm. Fractions indicated by the *solid bars* were pooled and used for further separation by reversed-phase HPLC. Positions of cysteine-containing tryptic peptides are indicated in Table 1.

Modification of buried cysteine residues with [^{14}C]-iodoacetamide in the presence of 6 M GdHCl was performed after pre-incubation of phytochrome as Pfr in 20 mM Tris-HCl buffer, pH 7.8, containing 2 mM EDTA with 100 mM nonradioactive iodoacetamide at room temperature for 1 h.

Search for Potential Disulfide Bond Sites. Phytochrome (20 nmol, isolated without DTT present in the buffer for gel-filtration chromatography on Toyo-Pearl HW-65) was treated with 100 mM nonradioactive iodoacetamide in 200 mM Tris-HCl buffer with 6 M GdHCl and 2 mM EDTA (pH 8.3). Following reaction for 1 h at room temperature, excess reagent was removed by gel filtration in 6 M GdHCl. The phytochrome fraction was treated with 20 mM DTT at 37 °C for 2 h and concentrated to a final volume of 0.5 mL. Excess DTT was removed by gel-filtration chromatography in 200 mM Tris-HCl, 6 M GdHCl, 2 mM EDTA, and 0.1 mM DDT, pH 8.3. The phytochrome fraction was treated with 5 mM [^{14}C]iodoacetamide (8800 cpm/nmol) for 30 min. The reaction was completed by adding iodoacetamide to a final concentration of 100 mM and by incubating for an additional 30 min.

Tryptic Digestion of Carboxamidomethylated Phytochrome. After dialysis against 100 mM ammonium bicarbonate, carboxamidomethyl phyA (CAM-phyA) was digested as a suspension of the protein (2−2.5 mL) for 5 h at 37 °C at a trypsin−phytochrome ratio of 1:50 (w/w). The digest was lyophilized and redissolved in 100 mM ammonium bicarbonate before fractionation by gel-filtration chromatography.

Separation of Tryptic Digests. The tryptic peptides were separated into 8 fractions by gel-filtration chromatography (Bio-Gel P-6, 1.5 × 95 cm; with 100 mM ammonium bicarbonate, pH 8.3 at 4 °C) at a flow rate of 10.4 mL/h with detection at 280 mn. Fractions (II−VII, Figure 1) were freeze-dried and subjected to reversed-phase HPLC [Vydac 218TP54 column; 0.46 × 25 cm; solvent A, water, 0.1%

trifluoroacetic acid (TFA); solvent B, 90% acetonitrile, 0.1% TFA]. Peptides were eluted from the reversed-phase column using 100% solvent A for 6 min, followed by a gradient to 20% solvent B in 45 min, to 25% solvent B in 10 min, to 50% solvent B in 25 min, to 100% solvent B in 10 min, and finally holding at 100% solvent B for 10 min. Peptides were detected by their absorbance at 216 nm.

Electrospray Ionization Mass Spectrometry (ESIMS). Fractions collected following reversed-phase HPLC were dried and redissolved in 0.1% TFA. The molecular weights of the peptides in these fractions were determined via direct injection ESIMS using a Micromass Platform II quadrupole mass spectrometer. Aliquots of the peptide solution were injected directly into a 5 mL/min flow of 50% acetonitrile. Molecular masses of peptides were routinely determined with an uncertainty of less than 0.3 Da. Some peptides were identified from their collision-induced dissociation (CID) spectra. The CID spectra were obtained following dissociation by using different sample cone voltage (*24*) before the skimmer in the quadrupole mass spectrometer, or using a Micromass Autospec magnetic sector mass spectrometer equipped with an orthogonal acceleration time-of-flight analyzer (*25*).

Reactivity of Cysteine Residues. The reactivity of Cys residues in phyA under native conditions (as Pr and Pfr, duplicate experiments each) was determined as the quantity of [^{14}C]-label (in thousands of cpm) bound to a cysteine residue per normalized quantity of the peptide. Briefly, each cysteine-containing peptide (500−3000 pmol) was subdigested under the conditions which ensured quantitative analysis of the labeled fragment by reversed-phase HPLC. Peptides T34, T36, T81, and T83 were digested with 40 μL of pepsin solution ($A_{280} = 0.025$) in 2% formic acid for 2.5 h at room temperature. The digestion time for peptides T10, T14, T16, T29, T73, T74, T78, T86, and T88 was 12 h. For analysis of cysteine-containing fragments of peptide T98, digestion of gel-filtration fraction II (derived from 6−10 nmol of total tryptic digest of CAM-phyA) was performed for 12 h using 60 μL of pepsin solution with $A_{280} = 0.05$. Peptide fractions T26, T47, and T96 were treated with 20 ng of Asp-N protease in 30 μL of 20 mM ammonium bicarbonate for 14 h at room temperature. Peptide T100 was digested with 40 μL of subtilisin solution ($A_{280} = 0.04$) in 20 mM ammonium bicarbonate for 12 h. These digestion conditions resulted in cysteine-containing fragments which eluted on HPLC profile as pure peptides. The relative quantity of each labeled fragment (Q_x) was determined using peptic fragment FGWCSEW of the tryptic peptide T78 as the reference according to the following equation:

$$Q_x = S_x\epsilon_{st}/S_{st}\epsilon_x$$

where S_{st} and ϵ_{st} are the HPLC peak areas of the reference fragment FGWCSEW and its extinction coefficient (79 470 M $^{-1}$ cm^{-1}); S_x and ϵ_x are the peak area and the extinction coefficient for the analyte fragment, respectively. The extinction coefficient for each Cys-containing fragment was determined from the known sequence of the fragment as a sum of absorbances of the peptide bonds, aromatic amino acid residues, methionine, and carboxamidomethyl cysteine. Extinction coefficients of Trp, Tyr, Phe, His, Met, and carboxamidomethyl cysteine (at 216 nm) are taken as 35 290, 6675, 4410, 5000, 800, and 800 (M^{-1} cm^{-1}), respectively (*26*). The extinction coefficient for a peptide bond at 216 nm (630 M^{-1} cm^{-1}) was determined by using dipeptide Gly-Gly as a standard. The relative reactivity values for all cysteine residues, in terms of the ratio of the radioactivity of the labeled fragment to its normalized quantity, were determined by setting the reactivity of Cys-311 for Pfr equal to 100.

RESULTS

The total tryptic digest of CAM-phyA was completely mapped using various mass-spectrometric methods after separation of the digest by gel-filtration chromatography on a Bio-Gel P-6 column followed by reversed-phase HPLC. Mass-spectrometric analysis of the peptides showed that phyA3, phyA4, and phyA5 isoforms (*19*) are present in phyA preparations isolated from etiolated oat seedlings. More than 80% of the phyA pool was represented by two very closely related isophytochromes, A5 and A3 (unpublished results). The sequence analysis of isolated peptides allowed us to confirm more than 97% of the sequence of phyA3 isoform. All cysteine-containing peptides were identified on the map.

[^{14}C]Iodoacetamide was used for specific modification because the extent of modification could be easily followed by determination of radioactivity of the peptides. The tryptic peptides were tentatively identified by matching their molecular masses with the masses expected from tryptic cleavage of the amino acid sequence of phyA3 as deduced from its cDNA (*19*). Table 1 shows the amino acid sequences of the peptides used in the present studies and the methods which confirmed the identities of cysteine-containing peptides of phyA. Most of the Cys-containing peptides resolved by gel filtration and reversed-phase HPLC were found to be pure according to ESIMS analysis. However, several radioactive fractions were a mixture of two and more peptides that interfered with quantitative determination of the cysteine-containing peptides. In addition, each of the tryptic peptides T26 and T98 contained two cysteine residues, which could have different reactivity. To ensure quantitative determination of the [^{14}C]-label incorporation, additional subdigestions of labeled peptides were used for identification of each Cys residue. It is noted that some Cys-containing regions were represented by two or more different peptides. For instance, two different peptides were obtained for Cys-311-, Cys-715-, Cys-850-, and Cys-869-containing regions, each as a result of "chymotryptic" activity of trypsin or incomplete tryptic cleavage of some specific peptide bonds. Tryptic fragments 445−455 (with Cys-445) and 932−945 (with Cys-939) were also found in two fractions each as a result of cyclization of N-terminal carboxamidomethyl cysteine and glutamine, respectively. In Table 1, only those peptides which provided more definite identification of the modification sites are shown. Modification with iodoacetamide under conditions used in the present studies was specific to Cys residues: No Met or His modification was observed.

Reactivity of Cys Residues in Oat PhyA under Native Conditions. Modification of the Pr and Pfr forms of phyA under native conditions (20 mM Tris-HCl buffer, pH 7.8, molar ratio phytochrome−iodoacetamide of 1:30, 0 °C) showed approximately 10% increased labeling of Pfr as

Table 1: Sequences, Molecular Weights, and Gel-Filtration Elution Pattern of Cysteine-Containing Peptides of Oat PhyA

peptide[a]	sequence[b]	found MH^+ [c]	bio-gel fraction	sequence confirmation[d]
T10	77LIQTFGCLLALDEK[90]	1621.2	IV	Pep
T14	[142]ALGFADVSLLNPILVQCK[159]	1958.6	III, IV	Pep
T16	[172]ATGCLVVDFEPVKPTEFPATAAGALQSYK[200]	3069.5	II, III	Pep, Glu-C
T19	[209]IQSLPGGSMEVLCNTVVK[226]	1961.8	III, IV	Pep, Glu-C
T26	[287]MICDCR[292]	854.5	VII	CID ESIMS
T29	[298] VIEAEALPFDISLCGSALR[316]	2061.6	III	Pep
T34	[364]LWGLLVCHHESPR[376]	1733.0	VI	Pep
T36	[384]YACEFLAQVFAVHVNR[399]	1923.9	VI	Pep
T43	[445]CDGAALLYGGK[455]	1123.5	VI	CID ESIMS, Ch
T47	[481]DSTGLSTDSLHDAGYPGAAALGDMICGMAVAK[512]	3154.5	II	Pep, Ch, Asp-N
T73	[704]DDGPVILVVNACASR[718]	1586.6	IV	Pep
T74	[719]DLHDHVVGVCFVAQDMTVHK[738]	2306.1	III	Pep
T78	[753]AIIHNPNPLIPPIFGADEFGWCSEWNAAMTK[783]	3513.9	III	Pep, Glu-C
T81	[796]MLLGEVFDSSNASCPLK[812]	1867.2	III, IV	Pep, Edm
T83	[815]DAFVSLCVLINSALAGEETEK[835]	2365.4	III	Pep
T86	[847]YIECLLSANR[856]	1238.6	VI	Pep
T88b	[869]CFIHVASHELQHALQVQQASEQTSLK[894]	2991.0	III	Pep, Glu-C
T96	[932]QIHVGDNCHHQINK[945]	1698.8	V	Asp-N, Edm
T98	[959]SSCLDLEMAEFLLQDVVVAAVSQVLITCQGK[989]	3424.2	II	Pep
T100	[993]ISCNLPER[1000]	988.6	VI	Sub

[a] Peptides are numbered as they appeared in the sequence. The peptides contained Cys residue(s) modified with iodoacetamide. [b] Amino acid sequences based on phyA3 cDNA sequencing (Hershey et al., 1985). [c] The mass $[M + H]^+$ as determined by ESIMS. [d] The following methods were used to confirm the peptide assignment: Edm, N-terminal Edman sequencing; CID ESIMS, collision induced dissociation electrospray ionization mass spectrometry; and Sub, Pep, Ch, Asp-N, and Glu-C, subdigestions of corresponding peptides with subtilisin, pepsin, chymotrypsin, Asp-N protease, and Glu-C protease, respectively. The subdigestions were followed by ESIMS.

compared to Pr. The total number of SH groups modified in Pfr was 1.8/phytochrome monomer after 60 min of the reaction. These conditions represent a compromise between the desire to achieve substantial labeling of the protein to analyze the reactivity of as many individual cysteine residues as possible in phyA and the necessity to avoid saturated conditions for any cysteine residue. The increased extent of modification eliminated differences in the extent of the modification of Pr and Pfr (data not shown).

Identification of the sites of the label attachment was performed by tryptic mapping of the modified Pr and Pfr forms of phyA. Initially, the digests were applied to a Bio-Gel P-6 column. A typical gel-filtration profile of total tryptic digest of CAM-phyA is shown in Figure 1. Figure 2 illustrates the radioactivity distribution among the fractions of tryptic digest of modified Pfr. Gel-filtration fractions I and VIII had low radioactivity and were not analyzed. Fractions II−VII were further separated by reversed-phase HPLC. More than 80% of the total radioactivity eluted from the gel-filtration column was found in three fractions: III, IV, and VI (see Figure 2). Preliminary direct HPLC of fraction II which contained four large cysteine-containing peptides (T16, T47, T88, and T98) showed that labeling was mainly due to modification of cysteine(s) of T98. However, the yield of this hydrophobic peptide was low, and only 15% of the radioactivity injected to the column was recovered. More than 55% of the radioactivity was recovered after predigestion of fraction II with pepsin. The major labeled peptide on the HPLC profile of the peptic digest (not shown) represented the N-terminal fragment SSCL (residues 959−962) of T98, whereas the C-terminal peptic fragment ITCQGK (residues 984−989) had very low radioactivity.

Data on the total amount of radioactivity found in HPLC fractions after separation of gel-filtration fractions III−IV (see Figure 2) indicated that most of the radioactivity was found in fractions containing peptides T29, T81, T78, T73, and T14, with corresponding modification sites at Cys-311, Cys-809, Cys-774, Cys-715, and Cys-158. Only the peptides corresponding to two modification sites, Cys-995 and Cys-850 (peptides T100 and T86), were obtained after separation of gel-filtration fractions V and VI. A small amount of radioactivity found in gel-filtration fraction VII was due to the presence of peptide T86 and to slight labeling of T26. Very similar data were obtained after separation of the gel-filtration fractions of tryptic digest of the iodoacetamide-modified Pr form of phyA. The yield of different tryptic peptides could differ significantly, so accurate data on the relative reactivity of Cys residues could be obtained by comparing the quantities of the label attached to each Cys residue per normalized quantity of the peptide. Isolated radioactive peptides were additionally subdigested with appropriate enzyme to ensure reliable quantitative analysis of the labeled fragments by reversed-phase HPLC. The subdigestions indicated in Table 2 allowed us to determine the relative quantities of the labeled fragments (see the Materials and Methods section) because they eluted highly purified. Table 2 summarizes the reactivity results for cysteine residues of the Pr and Pfr forms of phyA toward iodoacetamide under native conditions. The reactivity/accessibility of Cys-311 in the Pfr form, highest among the cysteine residues in oat phyA, was taken as 100.

Only two Cys residues of the chromophore domain showed a significant reactivity under native conditions: Cys-158 and Cys-311. The Cys-311 residue is near the chromophore attachment site (Cys-321). Surprisingly, Cys-311 modification did not cause any significant change in the spectral properties of the chromoprotein (under these conditions the total number of modified Cys for Pr was 1.6 residues/monomer and at least 30% of Cys-311 was derivatized with iodoacetamide). Moreover, phytochrome preparations, modified with 100 mM iodoacetamide at room temperature for 30 min in 20 mM Tris-HCl buffer after removal of excess reagent, displayed essentially unperturbed photoreversibility of Pr and Pfr forms (data not shown). Cysteine-311 was

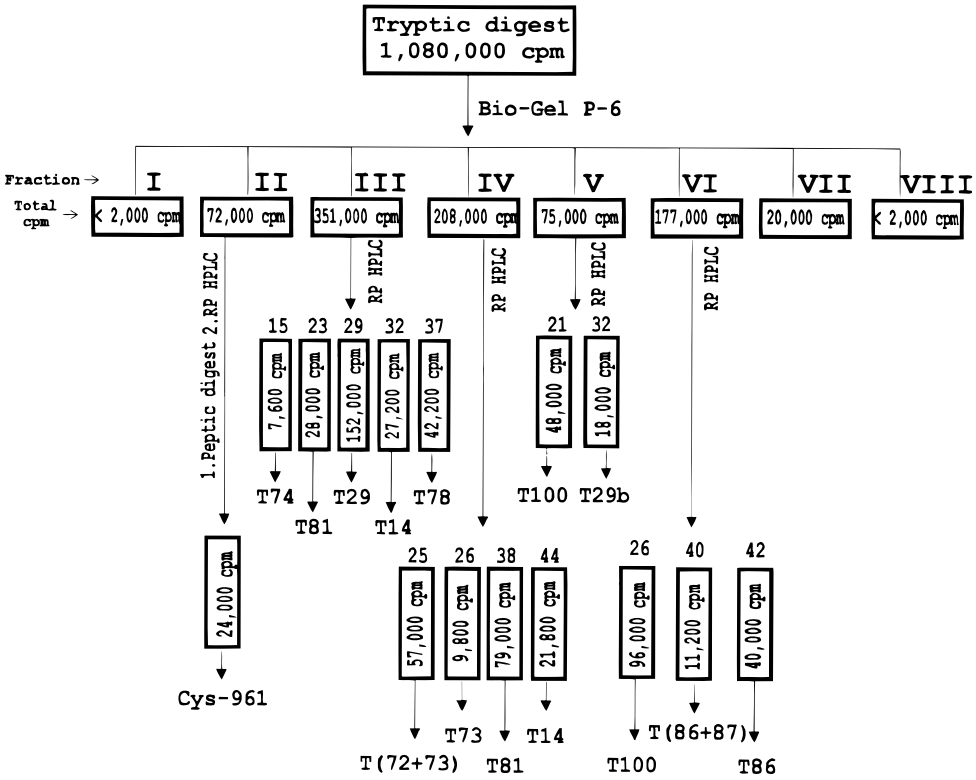

FIGURE 2: Schematic representation of analysis of [^{14}C] iodoacetamide incorporation after labeling of the Pfr form under native conditions. I−VIII are fractions obtained after separation on the Bio-Gel P-6. The total amount of the radioactivity is indicated in boxes. HPLC fractions are shown by arabic numerals (fractions with total radioactivity below 2500 cpm are not shown).

Table 2: Reactivity of Cys Residues of Oat PhyA toward Iodoacetamide under Native Conditions

cys residue	tryptic peptide/type of digest[a]	sequence[b]	reactivity[c]
Cys-83 (Pr, Pfr)	T10/pepsin	^{82}GCL^{84}	no[d]
Cys-158 (Pr)	T14/ pepsin	^{156}VQCK^{159}	13.5 ± 1.3
Cys-158 (Pfr)	T14/ pepsin	^{156}VQCK^{159}	13.7 ± 1.2
Cys-175 (Pr, Pfr)	T16/ pepsin	^{172}ATGCL^{176}	no
Cys-221 (Pr, Pfr)	T19/pepsin	^{221}CNTVVK^{226}	low[e]
Cys-289 (Pr, Pfr)	T26/Asp-N	^{287}MIC^{289}	low
Cys-291 (Pr, Pfr)	T26/Asp-N	^{290}DCR^{292}	no
Cys-311(Pr)	T29/pepsin	^{311}CGSALR^{316}	78.4 ± 5.5
Cys-311(Pfr)	T29/pepsin	^{311}CGSALR^{316}	100 ± 3.6
Cys-370 (Pr, Pfr)	T34/pepsin	^{369}VCHHESPR^{376}	no
Cys-386 (Pr, Pfr)	T36/pepsin	^{384}YACEF^{388}	no
Cys-445 (Pr, Pfr)	T43/pepsin	^{445}CDGALL^{450}	no
Cys-506 (Pr, Pfr)	T47/Asp-N	^{503}DMICGMAVAK^{511}	no
Cys-715 (Pr, Pfr)	T73/pepsin	^{711}VVNACASR^{718}	18.4 ± 3.6
Cys-728 (Pr, Pfr)	T74/pepsin	^{727}VCF^{729}	5.1 ± 0.4
Cys-774 (Pr, Pfr)	T78/pepsin	^{771}FGWCSEW^{777}	48.0 ± 2.4
Cys-809 (Pr, Pfr)	T81/pepsin	^{803}DSSNASCPLK^{812}	27.4 ± 2.4
Cys-821 (Pr, Pfr)	T83/ pepsin	^{818}VSLCVL^{823}	5.4 ± 0.5
Cys-850 (Pr, Pfr)	T86/pepsin	^{847}YIECL^{851}	19.3 ± 0.7
Cys-869 (Pr, Pfr)	T88b/pepsin	^{869}CFIHVASHELQHALQ^{883}	4.4 ± 0.4
Cys-939 (Pr, Pfr)	T96/Asp-N	^{937}DNCHHQINK^{945}	no
Cys-961 (Pr, Pfr)	T98[f]/pepsin	^{959}SSCL^{962}	18.1 ± 2.5
Cys-986 (Pr, Pfr)	T98[f]/pepsin	^{984}ITCQGK^{989}	low
Cys-995 (Pr, Pfr)	T100/subtilisin	^{993}ISC^{995}	59.2 ± 4.2

[a] Tryptic peptides and types of subdigests used for the analysis (Asp-N stands for Asp-N protease). [b] Cys-containing fragments analyzed by HPLC (with iodoacetamide derivatized cysteine). [c] Relative reactivity as determined in thousands of cpm per normalized quantity of the peptide (see Materials and Methods). The relative reactivity of Cys-311 in the Pfr form was taken as 100. [d] Radioactivity of the fraction was within background. [e] Relative reactivity was below 3.0. *Peptic digest was performed using gel-filtration fraction II containing T98.

the only Cys residue which showed a differential reactivity dependent on phototransformation (22% higher reactivity for Pfr than for Pr). More than half of the cysteine residues of the chromophore domain (Cys-83, Cys-175, Cys 291, Cys-370, Cys-386, Cys-445, and Cys-506) did not show any detectable labeling under native conditions in either Pfr or Pr form. Minor labeling was detected for Cys-221 in peptide T19 and Cys-289 in peptide T26, with the relative reactivity below 3% of that observed for Cys-311 in phyA as Pfr.

The reactivity of Cys residues in the C-terminal domain was not dependent on Pr → Pfr phototransformation. Six cysteines within this domain were readily modified under

native conditions: Cys-995, Cys-778, Cys-809, Cys-961, Cys-850, and Cys-715. Markedly lower reactivity was observed for Cys-728, Cys-821, and Cys-869, and only slight modification was observed for Cys-986 (Table 2). Only one cysteine residue (Cys-939) in the C-terminal domain was inaccessible to iodoacetamide under native conditions.

Potential Sites of S−S Bond Formation. Hunt and Pratt (*20*) reported that oat phyA contained two disulfide bonds. Our data showed additional incorporation of [^{14}C]iodoacetamide into the chromoprotein after treatment with DTT. Determination of the number of half-cystines modified (approximately 1.1/monomer) was lower than that required for a presumed S−S bond. To develop the scheme of isolation and identification of cystine-containing peptide(s), we determined potential Cys residues involved in S−S bond formation. For this purpose, oat phyA was extensively modified with 100 mM iodoacetamide in the presence of 6 M guanidine hydrochloride. The modified protein was reduced with DTT, and additional modification with [^{14}C]-iodoacetamide was performed. After tryptic digestion of the labeled phytochrome under standard conditions, sites of the label incorporation were determined using "two-dimensional" tryptic mapping. Of the total amount of [^{14}C]-label (210 000 cpm) applied to the gel-filtration column, 40 000, 39 000, 21 000, and 58 000 cpm were found in fractions II, III, IV, and VI, respectively. A typical gel-filtration profile of CAM-phyA tryptic digest is shown in Figure 1. Essentially no radioactivity was detected in fractions I and VIII. The HPLC of fraction II resulted in only a 15% recovery of total radioactivity applied to the column. However, 65% of the radioactivity was recovered after HPLC separation of total peptic digest of fraction II with a major labeled peptide SSCL corresponding to Cys-961 (18 000 cpm). Two labeled peptides were purified from fraction III: T29 and T78 (12 000 and 8400 cpm, respectively). Peptide T86 was identified as a major labeled peptide of fraction IV (5500 cpm). Most of the label of fraction VI was due to the modification of Cys-995 (peptide T100, total radioactivity 35 400 cpm). Fractions V and VII had low radioactivity (below 10 000 cpm) and no new additional labeled peptides as compared to peptides of fractions IV and VI were recovered from these fractions. Assignments of all major sites of the modification were confirmed by HPLC after subdigestion of the labeled peptides according to Table 2. Apart from these major sites of ^{14}C incorporation, most Cys residues showed minor amounts of the label.

Previous studies have shown that the dimer structure of oat phyA does not involve S−S bonding and that the N- and C-terminal domains are not linked by a disulfide bond (*11*, *14*). From the present study, we could not exclude the unlikely possibility of Cys-995 being involved in S−S bond formation with Cys-961 (see later). It is even less likely for Cys-995 to be linked to either Cys-774 or Cys-850. Only one site (Cys-311) was modified in the N-terminal chromophore domain after DTT reduction. Estimation of the extent of phyA modification at the most reactive site (Cys-995) showed that not more than 30% of this site became accessible for modification after the chromoprotein reduction. The other sites were much less accessible. It appears that the main sites of phytochrome labeling after reduction were Cys residues that were found to be most reactive under native conditions. Thus, additional modification of phyA by iodoacetamide after incubation of the chromoprotein with DTT can be explained by regeneration of SH groups from a partially oxidized protein. When 1 mM DTT was present in 20 mM Tris-HCl buffer at the last step of phytochrome purification, the freshly prepared phytochrome samples did not show any labeling in a similar experiment. It should also be pointed out that Cys-311, Cys-995, and Cys-774 were identified as the most reactive cysteines in oat phyA under native conditions when the chromoprotein was obtained without any reductant present at final purification steps.

Identification of Buried Cys-Containing Regions. To further probe the surface topography of phyA, we identified "buried" cysteines, which are accessible to chemical modification only after denaturation with 6 M GdHCl. For this purpose, the Pfr-phytochrome was treated for 1 h with 100 mM cold iodoacetamide at room temperature to modify both highly reactive and "sluggish" cysteines. After removing excess iodoacetamide, the reaction with [^{14}C]iodoacetamide was carried out in the presence of GdHCl. Identification of the sites of [^{14}C]-label incorporation was performed by tryptic mapping as described above. Tryptic peptides T10, T16, T26, T34, T36, T43, and T47 of the N-terminal domain and T96 of the C-terminal domain were the most strongly labeled peptides isolated from the tryptic digest. The label incorporation was confirmed by HPLC analysis after subdigestion of the peptides as indicated in Table 2. An HPLC analysis of Asp-N protease digest of labeled tryptic peptide T26, which has two closely positioned cysteines, showed that Cys-291 had a 14.5-fold higher label than Cys-289. This result is consistent with the data for phytochrome labeling under native conditions where a slight modification of Cys-289 (but not Cys-291) was observed following reaction of phyA with iodoacetamide. In a similar manner, peptic digestion of the gel-filtration fraction II containing peptide T98 showed no labeling for Cys-961, but detectable labeling of Cys-986. It should be noted that cysteine residues, which became accessible to iodoacetamide only after phytochrome denaturation, showed essentially the same values for [^{14}C]-label incorporation per unit quantity of peptide (data not shown).

DISCUSSION

Cysteine residues play an essential role in folding the chromoprotein domains. Among 23 cysteines of oat phyA, a majority of the cysteines are conserved among phytochrome A's from different plants. Residues corresponding to Cys-221, Cys-291, Cys-321, Cys-715, and Cys-939 of oat phyA are totally conserved in 23 known complete sequences of phytochromes A−E from higher plants as well as phytochromes from *Ceratodon*, *Selaginella*, and *Physcomitrella* (see ref *27* for sequence alignment).

Cys-321 is Linked to Phytochromobilin. Several highly conserved cysteine residues could be involved in S−S bond formation. Our results showed, however, that no disulfide bonds were present in oat phyA, as all 22 half-cystines were available for modification without reduction. A complete in vitro reduction of putative S−S bonds that might have been present in vivo seems very unlikely, as the buffers for phytochrome purification contained mercaptoethanol at a low concentration and the last step of phyA purification (gel-filtration chromatography) was performed without any reductant. Additional modification of phyA isolated without

the protection of SH groups after its reduction with DTT was related to the regeneration of SH groups from partially oxidized cysteine residues, mainly Cys-995, Cys-961, Cys-311, and Cys-774. Previously published data on the number of S−S bonds in phyA (*20*) also may be explained by partial oxidation of the reactive cysteines taking into account that reductants were omitted during phytochrome preparation. We can expect that folding of the native structure of other phytochrome species does not require disulfide bonds.

Topography of the Chromophore Domain. Data on the relative reactivity of cysteine residues allow us to draw some conclusions on the phyA conformation and the possible role of some other cysteine-containing regions. The chromophore domain contains determinants for photosensory specificity of the photoreceptor, for example, phyA versus phyB (*4, 6*). Information on the surface topography of the chromophore domain is available mainly from limited proteolysis studies. It is known that the 6-kDa N-terminal fragment of oat phyA is readily cleaved in the Pr form (but not in the Pfr form) by different proteases (*9*−*11, 14*). In the Pfr form, a surface-exposed region near the chromophore site, peptide bond Glu_{353}-Gln_{354}, was identified by Grimm et al. (*10*). Also, the structure of the N-terminal/chromophore domain is more compact than the C-terminal domain. Only two of the eleven Cys residues of the chromophore domain were accessible to iodoacetamide. On the other hand, only two of the eleven cysteines of the C-terminal domain were unmodified under native conditions. These data are consistent with the results of a limited proteolysis of oat phyA (*11, 14*). Although microenvironmental factors could significantly influence the iodoacetamide reactivity of SH groups under native conditions, surface accessibility for the neutral reagent is considered to be the major factor affecting the Cys reactivity (*28*).

Figure 3 schematically shows the molecular topography of oat phyA based on the present data and pertinent information from the literature. The regions around seven cysteine residues of the N-terminal domain, Cys-83, Cys-175, Cys-291, Cys-370, Cys-386, Cys-445, and Cys-506, are most likely buried, and the modification of these residues could be achieved only after phytochrome denaturation. The region 364−395 with a high degree of sequence homology among phytochromes, which includes Cys-370 and Cys-386, is apparently involved in folding the core of the chromophore domain (*30*). This region follows the cluster of four lysine residues (residues 360−363) which is resistant to trypsinolysis under native conditions (*10, 11*). Secondary structure predictions showed that the regions around Cys-370 had a very high probability of being a buried β-sheet (*27*).

Two closely positioned cysteine residues Cys-289 and Cys-291 showed different reactivities toward iodoacetamide. This region is also not readily accessible to the reagent. Cysteine-289 reacted slowly under native conditions, whereas the Cys-291 modification required phytochrome denaturation. These differences in reactivity could be explained by their different extents of submersion into the protein core as well as by their different microenvironments. These two cysteines are surrounded by charged amino acid residues, mainly Lys and Arg. It would be interesting to compare the reactivity of these two residues toward iodoacetamide and other SH-specific reagents to probe the local environment in this region. The sequence corresponding to Asp_{290}-Cys_{291} of oat phyA is conserved in all phytochromes (*27*), whereas Cys-289 is replaced by a hydrophobic residue in phytochrome B's. Most likely, the conserved residues of this region cannot be directly involved in the protein−protein interactions either in Pr or in Pfr, but these residues are probably necessary for the tertiary structure of the photoreceptor.

Two previously published papers on comparative analysis of the kinetics of SH group modification of the Pr and Pfr forms of large phyA (lacking the N-terminus) showed to a certain extent different results (*31, 32*). In later experiments, specific modification of SH groups of the Pr and Pfr forms of native oat phyA by several sulfhydryl reagents indicated that phototransformation resulted in exposure of additional SH groups in the chromophore domain (*21, 22*). Differences in the total number of cysteine residues modified in the Pr and Pfr forms of oat phyA were observed at early steps of SH group modification with 5,5′-dithiobis (2-nitrobenzoic acid), *N*-(1-pyrenyl)-maleimide, and *p*-chloromercuribenzoic acid. However, other reagents did not show any differences between Pr and Pfr (*21*). Our results showed that, of the two accessible cysteines Cys-158 and Cys-311, only the reactivity of Cys-311 was affected by phototransformation. The reactivity of Cys-311 was reproducibly 22% higher in the Pfr form than in the Pr form. The Cys-311 residue in the hydrophobic segment 308−316 is in the vicinity of the chromophore linkage, and it apparently occupies a position on or near the surface of the chromoprotein in both photochromic forms. This residue, which is the most reactive cysteine in oat phyA, was partially oxidized if DTT was not used during purification.

Smith and Cyr (*22*) suggested that modification of highly reactive SH groups of phyA resulted in a spectral shift of the Pr absorbance maximum. On the contrary, Eilfeld et al. (*21*) came to the conclusion that highly reactive SH groups are not directly involved in the photochemistry of the phyA chromophore. Our results are more consistent with the data of Eilfeld et al. (*21*). It was unexpected, however, that the modification of Cys-311 did not have any detectable effect on the spectral properties of phyA. By comparative analysis of the chromophore environments between C-phycocyanin and phytochrome, Partis and Grimm (*33*) suggested that the residue corresponding to Ser-309 of oat phyA could interact with the nitrogen of the phytochromobilin ring A. Cys-311 is close to the chromophore-linked Cys-321. Our results clearly showed that Cys-311 does not directly interact with the chromophore since its modification apparently does not perturb the chromophore environment. Also, replacement in the same region of the Asp-309 of pea phyA (corresponding to Asp-307 of oat phyA) with Asn or His caused no spectral changes in the chromoprotein (*34*). However, the reactivity of Cys-311 did increase upon Pr → Pfr phototransformation. This suggests that the chromophore isomerization (*35*−*38*) and movement (*39*−*41*) induce a conformational change around the Cys-311 site in oat phyA.

The Cys-158 residue also showed a significant [^{14}C]-labeling, though its reactivity was approximately 6 times lower than that for Cys-311. The Cys-158, however, did not show any partial oxidation found for several cysteine residues from the C-terminal domain with similar reactivity values toward iodoacetamide. This Cys residue is conserved in all phyA's. In phyB's, it is replaced by a serine. Most likely, Cys-158 is not fully exposed on the protein surface

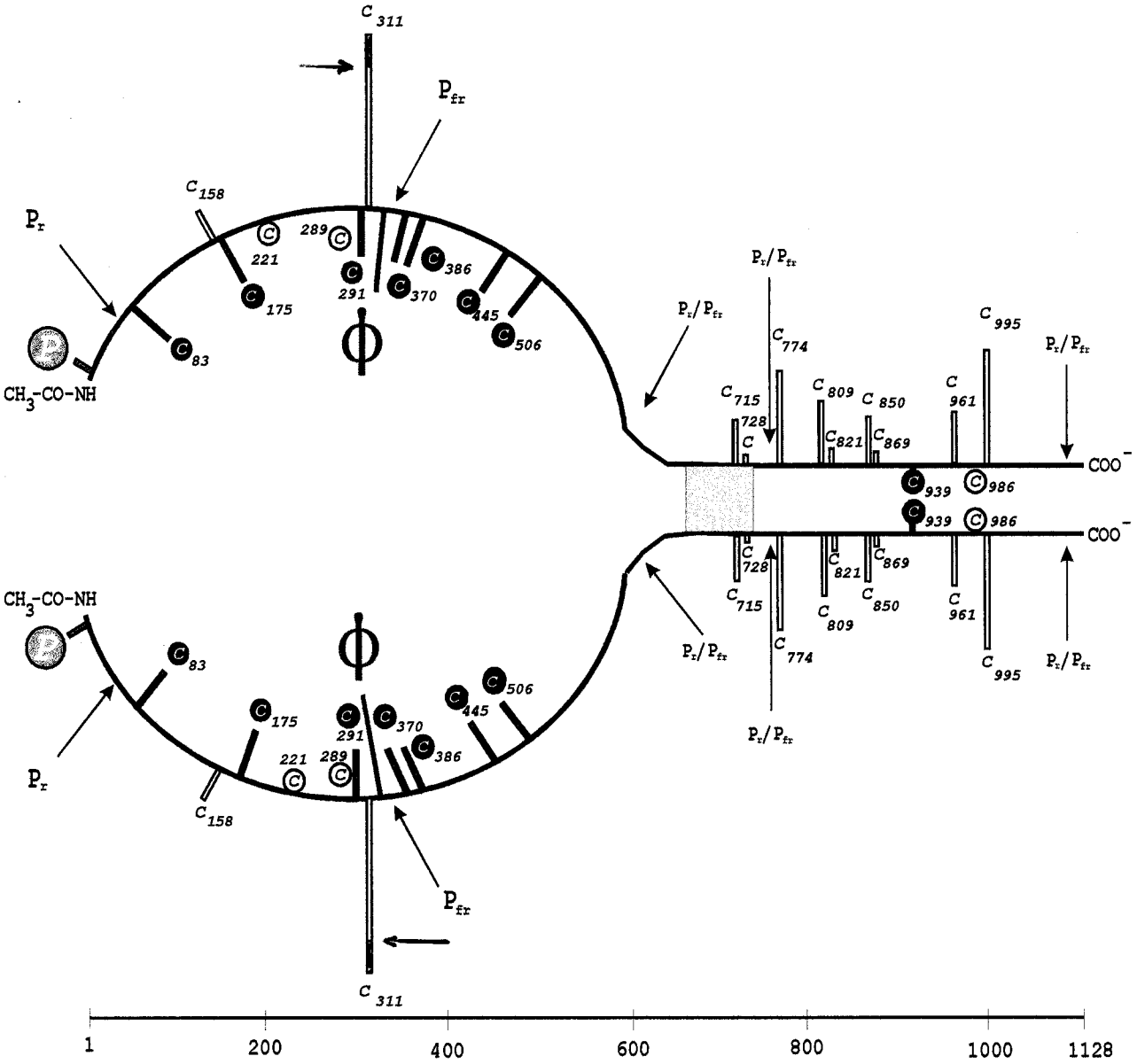

FIGURE 3: Schematic drawing for the molecular surface topography of oat phyA. Exposed cysteine residues are shown by open rectangles, where the height of the rectangles correlates with the relative reactivity of the cysteine residues. The increase of the reactivity of Cys-311 in Pfr (as compared to Pr and indicated by a horizontal arrow) is shown by solid black rectangles. Buried cysteines are shown in solid circles with black bars to the inside. Partially buried, slowly reacting cysteines are shown in open circles. Note that most of the Cys residues in the N-terminal domain are buried, reflecting the globular fold of each chromophore-containing subunit. In contrast, those in the C-terminal domain are exposed, indicating an open/extended conformation along the C-terminal domain. The encircled P indicates in vivo phosphorylation at Ser-7 (*29*); ϕ indicates phytochromobilin, covalently attached to Cys-321. Accessible sites for early proteolytic cleavage of native phyA in only Pr, or Pfr, or both Pr and Pfr (Pfr/Pfr) are indicated by arrows. A region of the C-terminal domain involved in regulatory activity (*4*) is stippled. Cysteine residues 221, 291, 321, 715, and 939 are totally conserved in 23 complete phytochrome sequences (see ref *27*). In addition, cysteines 774, 809, and 869 are conserved in phyAs and phyBs [and in more than 70% of all sequences (*27*)]; cysteines 83, 158, 311, 506, and 850 are conserved in phyAs.

and is apparently less accessible to the reagent than Cys-311 is. The region corresponding to residues 150−200 of oat phyA was proposed to play a role as a determinant of the photosensory specificity of phyA versus phyB (*27*). Cys-158 and Cys-311, which are totally conserved among phyA's from various plants, may be involved in the signaling functions specific for phyA.

Apart from Cys-291 and Cys-321, the N-terminal domain of phyA has one more cysteine residue (Cys-221) totally conserved in all known phytochrome sequences. Cys-221 was found to react slowly under native conditions, and it is apparently shielded. A comparative analysis of the reactivity of this residue in Pr and Pfr forms toward other SH-specific reagents would be of interest.

Topography of the C-Terminal Domain. The C-terminal domain is required for the biological activity of phytochromes (*15*, *16*). A short segment corresponding to oat phyA residues 714−731 of the proximal end of the C-terminal domain is required for effective transduction of the light signal to downstream components of the phytochrome signaling system (*4*, *6*). WD-40 repeats are recognized in many phytochrome sequences in the N-terminal half of the C-terminal domain (*27*, *42*, *43*). These motifs are known to play an important role in protein−protein interactions in

many regulatory proteins (*44*). Also, the distal half of the C-terminal domain has a sequence similar to that of the sensor histidine kinase module of bacterial two-component signaling systems (*45*). This module is present in phytochrome-like protein cph1 from cyanobacterium *Synechosystis* sp. PCC6803 (*46*). It has been suggested that cph1 is a functionally active light-regulated histidine kinase (*47*).

Results of phyA modification with iodoacetamide under native conditions showed that the phyA sequence from 715 to 850 contains several regions which are most likely positioned on or near the surface in the native chromoprotein, namely those regions around Cys-715, Cys-774, Cys-809, and Cys-850 (Figure 3). Of special interest is the high reactivity of Cys-715, which is conserved among all members of the phytochrome family. This residue is positioned in the regulatory sequences of phyA and phyB (*4*). Highly probable surface exposure of the Cys-715 may indicate direct involvement of this residue in the phytochrome signaling. For example, it could serve as a part of the molecular recognition module for putative regulator component(s). Clusters of high sequence homology around the highly reactive Cys-774 and Cys-809 (conserved in all phytochromes A and B) also suggest functional importance of this region. The Cys-774 residue is located in the region where Pea-25 monoclonal antibody binds. This antibody cross-reacts with phytochromes from a number of plants (*48, 49*). In this region, an exposed site near Lys-752 (or Lys-744) was identified by limited proteolysis (*10, 14, 50*).

The distal part of the C-terminal domain of oat phyA (residues 868−1100) having modest sequence homology with bacterial sensor histidine kinases (*45*) contains five cysteine residues. By limited proteolysis, Grimm et al. (*9, 10*) identified the peptide bond Arg_{1092}-Asn_{1093} as the surface site in this module. Highly reactive Cys-995, the most easily oxidized Cys residue in oat phyA, is apparently positioned on the surface of the chromoprotein. The amino acid sequence of the Cys-995-containing region is the most variable one among all cysteine-containing sequences of oat phyA. The two cysteines of tryptic peptide T98 (residues 959−989) showed differential labeling with iodoacetamide. The Cys-961 may be included in a group of highly reactive cysteines, whereas only partial labeling of Cys-986 was achieved at high concentrations of iodoacetamide. The highly reactive Cys-961 is surrounded by charged amino acid residues and is located most likely on or near the surface. On the contrary, Cys-986 is positioned in a long stretch of hydrophobic amino acid residues (residues 974−988) and is presumably significantly less accessible to iodoacetamide. "Masking" of SH groups by their involvement in hydrophobic interactions is well-documented (*51*−*53*). Relatively low reactivity of Cys-869 as well as Cys-728 can also be explained by hydrophobic masking of these residues. The amino acid sequence around Cys-939 is variable to a significant extent, but this residue itself is conserved in different types of phytochromes. Cys-939 is the only cysteine residue of the C-terminal domain that is totally inaccessible to iodoacetamide unless the phytochrome is denatured. This residue most likely plays a significant role in folding the C-terminal domain.

As indicated earlier, the conformational changes induced by Pr → Pfr phototransformation are clearly identified in the N-terminal domain of phyA. It is generally accepted that the conformational changes also occur in the hinge region (around residues 596−600 of oat phyA) and around peptide bond Lys_{752}-Ala_{753} (or Lys_{743}-Phe_{744}) (*9, 10, 14, 50, 54*). The Pr- and Pfr-dependent conformational changes around Trp-733 and Trp-777 were suggested from tryptophan fluorescence quenching experiments (*55, 56*). The possible conformational differences between Pr and Pfr near the C-terminus of pea phyA have also been suggested (*57, 58*). However, the present results did not reveal any differences in accessibility and/or microenvironment between Pr and Pfr. Thus, the present method may be inadequate to address the possible interdomain cross-talk between the N- and C-terminal halves of the phytochrome molecules.

Comparison of the accessibility of the C-terminal domain (in Pr and Pfr) to time-dependent proteolysis is significantly complicated by the differences between conformations of the extreme N-terminus of phyA in the Pr and Pfr forms. Thus, a 83-kDa tryptic fragment derived presumably from the cleavage at Lys_{752}-Ala_{753} peptide bond is observed only in Pfr (*10*), but it cannot be detected in Pr due to fast cleavage of the N-terminal 6-kDa peptide. Our previous data also showed that Lys_{752}-Ala_{753} peptide bond is readily cleaved under mild conditions of limited trypsinolysis in both Pfr and Pr (*59*). Here, the cleavage was monitored by the formation of a 38.5-kDa fragment by immunoblot with C-terminus-specific antibodies Pea-25 (*48*). How the light-induced conformational changes in the N-terminal domain are transmitted to the C-terminal domain is important for understanding the interdomain signaling by phyA versus phyB (*56*).

In conclusion, selective modification of phyA with iodoacetamide revealed several regions which most likely are involved in folding the cores of the N- and C-terminal domains, namely the regions containing Cys-83, Cys-175, Cys-370, Cys-386, Cys-445, Cys-506, and Cys-939. Cys-291 is totally conserved in all known phytochrome sequences. It is likely to play a critical structural rather than functional role. Cysteine-311 and several cysteine residues of the C-terminal domain (Cys-995, Cys-715, Cys-774, Cys-809, Cys-850, and Cys-961) are most likely located on or near the surface of the chromoprotein. The surface-exposed and conserved Cys-715 and Cys-774 may be directly involved in protein−protein interactions common for all phytochromes. The region around Cys-311 may be involved in interactions with differential signal transduction components specific for phyA (vs phyB).

From Figure 3, it is not surprising that the C-terminal domain is extended and surface-exposed, since the regulatory motif there must be recognized by putative phytochrome receptors via protein−protein interactions. It remains to be seen how the interdomain signaling between the N- and C-terminal domain takes place to transmit phyA- and phyB-specific light signals to the regulatory motif or "Quail box" (*4*) for the downstream signaling pathway.

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