Phototransformation of Pea Phytochrome A Induces an Increase in α-Helical Folding of the Apoprotein: Comparison with a Monocot Phytochrome A and CD Analysis by Different Methods†

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ABSTRACT: The photoreversible conformational change associated with the $P_r \rightarrow P_{fr}$ transformation of a dicot phytochrome A (*Pisum sativum*, pea) has been probed by circular dichroism (CD) studies. Three different CD analysis methods have been used to determine the secondary structure of pea phytochrome A in both P_r and P_{fr} forms. We have shown that the secondary structure of dicot pea phytochrome A is very similar to the structure of monocot oat phytochrome A which was determined earlier [Sommer & Song (1990) *Biochemistry 29*, 1943–1948]. As with oat phytochrome A, an increase in the α-helical folding of the apoprotein takes place when photochrome in the P_r form is phototransformed to the P_{fr} form. This conformational change might well be a general characteristic of all phytochrome A's.

Phytochrome is a photochromic light receptor that controls many aspects of photomorphogenesis throughout a plant's life cycle [for a review, see Kendrick and Kronenberg (1994)]. Phytochrome is synthesized in the plant in its red-light-sensing P_r[1] form (λ_{max} = 660 nm). Red light reversibly yields the regulatorily active P_{fr} form (λ_{max} = 730 nm). Phytochrome is a homodimer (Lagarias & Mercurio, 1985; Jones & Quail, 1986), and each monomer is made up of two components: an apoprotein with a molecular mass of ≈120 kDa (Quail et al., 1991) and an open-chain tetrapyrrole covalently linked to it (Lagarias & Rapoport, 1980). The primary component(s) with which P_{fr} interacts remain(s) largely unknown, but elucidation of specific structural differences between the P_r and P_{fr} forms of phytochrome is crucial for understanding how P_{fr} triggers a wide variety of photomorphogenic responses including the transcriptional regulation of photosynthetic genes.

Conformational changes involved in the $P_r \rightarrow P_{fr}$ phototransformation of oat phytochrome A are well documented [e.g., see Lagarias and Mercurio (1985), Jones et al. (1985), Grimm et al. (1988), Cordonnier et al. (1985), Vierstra et al. (1987), Chai et al. (1987) and Sommer and Song (1990)]. The phototransformation of oat phytochrome A induces a conformational change along the N-terminal chain to an amphiphillic α-helix which selectively interacts with the chromophore in the P_{fr} form and occurs in a few milliseconds time scale (Parker et al., 1991, 1992; Chen et al., 1993). In the case of dicot pea phytochrome A, conformational differences between P_r and P_{fr} have been demonstrated by using proteolytic mapping studies (Nakazawa et al., 1993), by resonance Raman spectroscopy (Mizutani et al., 1993), and by differential affinity for various chromatographic materials (Yamamoto et al., 1981; Nakazawa et al., 1991).

Although phytochromes from monocot and dicot plants share relatively high amino acid homologies with apparently common spectroscopic and photochemical properties, there are significant structural differences between them, as revealed by peptide mapping (Nakazawa et al., 1993) and by the fluorescence dynamics of Trp residues (Wells et al., 1994). Here we describe the study of the photoinduced conformational changes in a dicot phytochrome A based on the UV circular dichroism (CD) spectra (187–240 nm) of its P_r and P_{fr} forms. The protein conformation is estimated from the CD data using three different computational methods to assign the structural differences between P_r and P_{fr}. The results are compared with the conformational differences detected for the monocot oat phytochrome (Chai et al., 1987; Sommer & Song, 1990). The data also indicate that the secondary structures of oat and pea phytochrome A's are similar. Further, the $P_r \rightarrow P_{fr}$ photoreversible change in α-helical content is conserved between oat and pea phytochromes A and thus likely conserved between monocots and dicots in general.

MATERIALS AND METHODS

Purification of Pea Phytochrome A. The purification of *Pisum sativum* phytochrome A was performed as described elsewhere (Tokutomi et al., 1988).

Circular Dichroism: Measurements and Analysis. All experiments were performed under a green safelight. The CD studies were carried out with a JASCO-600 spectropolarimeter flushed with N_2 and calibrated with *d*-10-camphorsulfonic acid as described by the manufacturer. All spectra were recorded at room temperature (≈23 °C) in a 1-mm quartz cell. Reproducibility of the CD and ΔCD ($P_{fr} - P_r$) spectra was good, as several scans for P_r and P_{fr} samples were respectively nearly superimposable with minor differences between spectra of different samples of phytochrome. The pea phytochrome preparation was passed over a buffer exchange DG-10 column (BioRad, Richmond, CA) equilibrated with 20 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.8. The concentration of pea phytochrome was determined using $\epsilon = 1.32 \times 10^5$ cm^{-1} M^{-1}

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[1] Abbreviations: CCA, convex constraint analysis; CD, circular dichroism; EDTA, ethylenediamine tetraacetic acid; kDa, kilodalton(s); P_r, red light absorbing fotm of phytochrome; P_{fr}, far-red light absorbing form of phytochrome; SAR, ratio of the absorbance at 660 nm versus the absorbance at 280 nm; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UV, ultraviolet.

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(Lagarias et al., 1986) at 660 nm. The SAR (ratio of the absorbance at 660 nm versus the protein absorbance at 280 nm) was 1.01. For each CD spectrum, three scans were averaged by the computer. The parameters on the instrument were as follows: scan speed, 20 nm/min; sensitivity, 20 mdeg; bandwidth, 0.1 nm; time constant, 1 s. Spectra of the P_r and P_{fr} forms of pea phytochrome A were recorded after photoconverting the phytochrome *in situ*. For the P_{fr} recordings, the sample was irradiated with red light for ≈20 s in between each scan to convert any dark-reverted phytochrome back to P_{fr}. CD spectra were recorded for two different phytochrome samples, and all recorded spectra were mathematically smoothed and averaged. The CD spectrum of P_r presented in Figure 1 is the average of spectra recorded before and after being cycled to P_{fr}.

Protein conformation calculations were performed with the CD data points taken between 187 and 240 nm at 1-nm intervals using (i) the Variable Selection Method (Manavalan & Johnson, 1987), (ii) Convex Constraint Analysis (Perczel et al., 1992; CCA software kindly provided by Dr. G. Fasman) with data points between 195 and 240 nm at 1-nm intervals, and (iii) the method of Yang et al. (1986) with data points as indicated in the table in order to allow a comparison with earlier published CD data for oat phytochrome (Chai et al., 1987; Sommer & Song, 1990). Manavalan and Johnson (1987) recommended that their method be used for CD data recorded down to 184 nm or lower. However, because of the high absorbance of our sample around 184 nm, which led to near-saturation of the photomultiplier tube on the instruments, we preferred not to include data below 187 nm. For the CCA analysis, the P_r and P_{fr} CD spectral data were appended to a reference data set of 24 globular proteins, and this CD data set was then deconvoluted.

Absorption Spectra and Phototransformation. Absorption spectra were recorded on a diode array spectrophotometer at room temperature. The wavelength resolution on this instrument is 2 nm. Phytochrome was photoconverted with a Cole-Parmer lamp equipped with an optical fiber, and red and far-red interference filters. The fluence rates were 11.67×10^{-3} and 1.21×1^{-3} W/cm^2 for red and far-red light, respectively. Gel electrophoresis was performed as described elsewhere (Laemmli, 1970).

RESULTS

The absorption maxima for the pea phytochrome used in our CD studies were at 666 nm for P_r and at 729 ± 1 nm for P_{fr}, and the SAR was 1.01. This indicates that the pea phytochrome is of high purity and proteolytically undegraded, which was confirmed by SDS–PAGE (data not shown). The photostationary concentration of P_{fr} in saturating red light was ≈81%. The CD spectra of P_r and P_{fr} in 20 mM potassium phosphate buffer containing 1 mM EDTA are shown in Figure 1. The two peak minima at 220–223 and 209 nm and the peak maximum at 195 nm indicate that phytochrome is predominantly α-helical in both P_r and P_{fr}. The spectra are in good agreement with data recorded earlier for oat phytochrome (Vierstra et al., 1987; Chai et al., 1987; Sommer & Song, 1990). The phototransformation from P_r to P_{fr} is accompanied by a CD spectral change with conservation of the typical α-helix peak positions. Photocycling back to P_r reverses this effect on the CD signal. This indicates that pea phytochrome A undergoes a reversible conformational change when phototransformed from P_r to P_{fr} and back. Structure calculations using the Variable Selection Method (Manavalan & Johnson, 1987) reveal that pea phytochrome is mainly

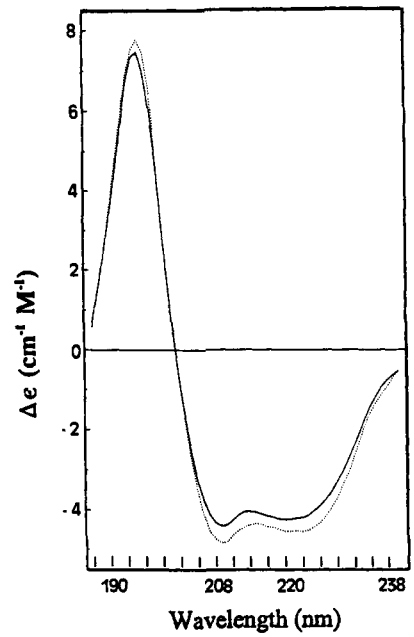

FIGURE 1: Far-UV CD spectra of native P_r (—) and P_{fr} (···) *Pisum sativum* phytochrome A in 20 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA. The spectra were recorded as described under Materials and Methods at 23 °C, mathematically smoothed, and averaged for two samples.

Table 1: Analysis of Secondary Structure Content of P_r and P_{fr} *Pisum sativum* Phytochrome A (CD Spectra in Figure 1) and *Avena sativa* Phytochrome A [Chai et al., 1987 (1); Sommer & Song, 1990 (2)][a]

protein	α-helix (%)	β-sheet (%)	β-turn (%)	random (%)
pea P_r[a]	43	11	29	17
pea P_{fr}[a]	48	5	31	15
187–240 nm				
pea P_r[b]	49	0.5	23.5	27
pea P_{fr}[b]	50.5	0	22.5	27
190–240 nm				
pea P_r[b]	45.5	10	13.5	31
pea P_{fr}[b]	50.5	0	16	33.5
200–240 nm				
pea P_r[b]	47.5	0	19	33.5
pea P_{fr}[b]	51.5	0	16.5	32
205–240 nm				
oat P_r[b]	51.2	0	24.3	24.5
oat P_{fr}[b]	54.5	0	21.5	24.0
190–240 nm	(1)			
oat P_r[b]	52.5	0	21.1	26.7
oat P_{fr}[b]	55.3	0	18.8	26.0
205–240 nm	(2)			

[a] The calculations were performed using the method of Manavalan and Johnson (1987). [b] Calculations were performed using the method of Yang et al. (1986). The data range was as indicated.

α-helical and that the α-helical structural content increases from 43% in P_r to 48% (corresponding to a 12% CD signal change at 222 nm) in P_{fr} (see Table 1). The root mean square error for the P_r calculations was ≥0.22 for P_r and ≤0.23 for P_{fr}. When the method of Yang et al. (1986) was used, the difference in α-helix content between P_r and P_{fr} was calculated to be 1.5–5% (corresponding to 3–11% CD signal change, respectively), depending on the lowest cutoff wavelength included for the analysis. We also analyzed the CD spectra using Convex Constraint Analysis as described (Perczel et al., 1992). The uniqueness of this method, compared to the other two methods employed here, lies in the fact that the pure component curves (the CD curves of typical secondary

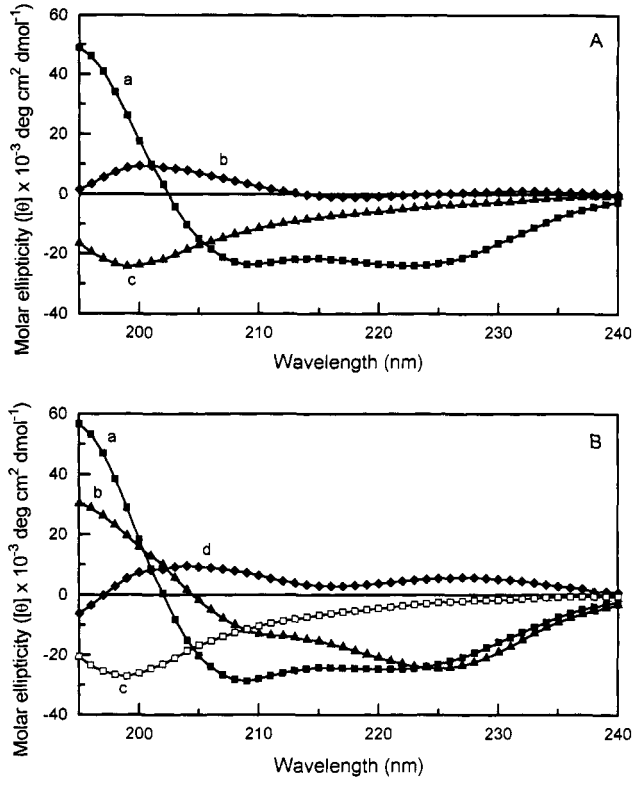

FIGURE 2: Pure component curves obtained by deconvolution of the circular dichroism curves of P_r and P_{fr} in Figure 1 by CCA analysis. (A) Number of allowed pure components is 3; (B) number of allowed pure components is 4. (a) α-Helix-like; (b) β-structure-like; (c) unordered like; (d) additional chiral contribution.

structural elements) are not calculated on the basis of X-ray diffraction data of a set of reference proteins but are extracted by deconvolution analysis from a set of CD data of both reference proteins and the protein(s) under study. Following the deconvolution process, the pure curves are then *assigned* to particular secondary structural elements such as α-helical conformation, random-coil conformation, etc., and the conformational weights (the percentages of pure secondary structural components) can be analyzed. The number of pure components (P) which are present in a particular conformational mixture must be estimated prior to deconvolution and are part of the input data. In four different deconvolution processes for the phytochrome CD spectra appended to a reference protein data set, we assumed 3, 4, 5, and 6 pure components P, respectively. The standard deviations were all acceptable and decreased with the number of pure components: $\sigma_{P=3} = 1.084$; $\sigma_{P=4} = 0.737$; $\sigma_{P=5} = 0.518$; $\sigma_{P=6} = 0.327$. For $P = 5$ or 6, the pure component curves contained duplicates (so-called "extra" pure components which showed close resemblance to the helix-like or β-structure-like pure component curves; Perczel et al., 1992), indicating that we had overestimated the number of pure components in the conformational mixture (Perczel et al., 1992). The results of the two deconvolutions with 3 and 4 pure components and the assignments of the pure components to secondary structural elements are given in Figure 2A,B. The pure component curves a and c are almost identical for both ($P = 3$ and $P = 4$) CCA analyses, and the λ_{max} and θ values are in both cases comparable to literature values for the α-helix-like and random-coil-like conformations (see Figure 2). The assignment of these pure component curves to the α-helix-like and random-coil-like conformation was therefore straightforward. The β-structure-like component for $P = 4$ shows characteristics of the β-turn conformation while the β-structure-like component for $P = 3$ is distorted (absence of minima and a low amplitude for the maximum). The assignment of this pure component to β-structure is therefore marginal. The weights of components for each secondary structural pure component for both P_r and P_{fr} are given in Table 2. The percentage α-helix-like and random-coil-like conformation is similar to what we have determined using the method of Yang et al. (1986) or Manavalan and Johnson (1987). Common to both CCA analyses is the increase in α-helix-like component by 1.3% with $P = 4$ (corresponding to a 3% change) to 4.5% with $P = 3$ (corresponding to an 8% change) when P_r is phototransformed to P_{fr}. The percentage range for this conformational change is very similar to what we have calculated with other methods (see Tables 1 and 2). When we increase the number of allowed pure components from $P = 3$ to $P = 4$, the analysis reveals a new pure component which we have labeled "other chiral contribution". This component may originate from twisted sheets or turns and non-peptide chromophores (aromatic residues, S–S bridges, or the bilin chromophore of phytochrome). The contribution of this component decreases by 5% when P_r is phototransformed to P_{fr} (Table 2).

Table 2: Percentages of Pure Secondary Structural Components for CCA Analysis of Pea Phytochrome A CD Spectra in Figure 1[a]

	$P = 3$		$P = 4$	
	P_r	P_{fr}	P_r	P_{fr}
α-helix-like (%)	55.3	59.8	41.8	43.1
β-structure-like (%)	22.0	17.0	18.4	21.3
random-coil-like (%)	22.6	23.2	21.2	22.0
other chiral contribution (%)			18.6	13.6

[a] The number of pure components, P, was 3 or 4.

DISCUSSION

The CD spectra of native *Pisum sativum* phytochrome A are shown in Figure 1. Approximately half of the amino acid residues in the phytochrome molecule assume α-helical conformation as calculated from mean residue ellipticities in the far-UV using three different computational methods (see Tables 1 and 2). The average α-helix content is slightly lower in pea compared to oat phytochrome A, but the overall secondary structure is very well conserved (see Tables 1 and 2).

We determined on average a 7% photoreversible change of the CD signal at 222 nm when P_r was converted to P_{fr} and *vice versa* (Figure 1). This change originates partly from an increase in α-helical structure (see Tables 1 and 2) as was consistently revealed by using three different calculative methods. The percentage of residues which adopt the α-helical conformation increases by 1.3–5% when Pr is phototransformed to P_{fr}. This compares closely to the 2.3–4% increase in α-helix calculated for oat phytochrome under identical buffer conditions (Chai et al., 1987; Sommer & Song, 1990). The sum of the β-structures displays in general a decrease upon P_r to P_{fr} phototransformation for both oat and pea phytochromes (with one exception for the CCA analysis with $P = 4$), but the absolute percentage varies considerably depending on the calculation method used. The random conformation component remains more or less invariant upon phototransformation. However, also for this conformational component, we note a large variability in terms of its absolute percentage, depending on the calculation method used (see Tables 1 and 2).

In the case of oat phytochrome A, it was shown that the photoreversible conformational change involves the extreme

N-terminal 6 kDa of the phytochrome polypeptide. When this part of the protein was bound to an antibody (Chai et al., 1987) or when it was proteolytically removed (Vierstra et al., 1987), the light-dependent change disappeared. Our attempts at analogous studies using truncated 114-kDa pea phytochrome A were hampered by the fast dark reversion of degraded pea phytochrome.

The molecular similarities between oat and pea phytochromes A which appear to be functionally identical are well documented: The overall amino acid sequence identity between pea phytochrome A and oat phytochrome A is 64–65% (Sato, 1988; Sharrock & Quail, 1989), and many of the amino acid substitutions are conservative substitutions. The resemblance in the hydropathy profiles shows a conservation of the general structure for phytochrome A (Sharrock & Quail, 1989; Quail et al., 1991). Romanowski and Song (1991) have proposed a folding into nine conserved domains for six phytochrome sequences from different plant species, monocot as well as dicot. Also, the hydrophobic moment profiles for the oat and pea phytochrome A N-termini are similar (Parker et al., 1991). Proteolytic mapping and spectral studies have further underscored the homology. Both in pea and in oat phytochromes, the N-terminus specifically interacts with the chromophore in the P_{fr} form. Truncation of the N-terminus leads to unaltered P_r spectra but results in blue-shifted P_{fr} spectra for both oat and pea phytochromes A. Several protease cleavage sites are similar between oat and pea phytochromes (Grimm et al., 1986, 1988), although a significant difference in tertiary structure between the two phytochromes is revealed when they are examined more closely (Nakazawa et al., 1993). However, both in pea and in oat phytochrome A, the N-terminus is proteolytically cleaved only in the P_r form at comparable sites (Grimm et al., 1988; Nakazawa et al., 1993).

Taken together, we propose that the N-terminus in pea phytochrome A undergoes a conformational change, to a more α-helical structure, in the P_{fr} form, analogous to what was observed for oat phytochrome A. Phytochrome domains which undergo a light-dependent conformational change are thought to be involved, directly or indirectly, in the triggering of the signal transduction which leads to photomorphogenesis. Recent work has demonstrated the functional importance of the N-terminal tail: Truncation of the 6-kDa N-terminal domain renders oat phytochrome A inactive in transgenic tobacco (Cherry et al., 1992), and mutation of the N-terminal serines to alanines increases the biological activity of rice phytochrome A in *Arabidopsis* (Stockhaus et al., 1992), apparently due to the increased amphiphilicity of the N-terminal α-helix (Furuya & Song, 1994).

Whether the N-terminal change in conformation between the red light-sensing P_r and the gene-regulating P_{fr} is restricted to the phytochromes of the A subfamily or whether it is a general feature of all phytochromes remains a question. A significant difference between the phyA and phyB sequences which stands out is the N-terminal extension of phytochrome B with 35–41 amino acid residues (Quail et al., 1991). The N-terminus is therefore a plausible candidate for differential recognition of the various phytochromes by the signal transduction chains, and thus the N-terminal conformation might allow distinction between phytochrome A and phytochrome B.

On the basis of the data presented in Tables 1 and 2, it is difficult to evaluate the relative merits of the three CD analysis methods. Qualitatively, all three methods suggest that both oat and pea phytochrome A molecules are predominantly α-helical and that a photoreversible change in α-helix accompanies the $P_r \rightarrow P_{fr}$ transformation. However, it is not possible to experimentally validate whether or not the 5–11% β-sheet structure is present in pea phytochrome P_r form but not in its P_{fr} form and in both forms of the oat phytochrome molecule (Table 1). The Manavalan and Johnson Variable Selection Method also yielded little or no β-sheet structure in oat phytochrome (Furuya & Song, 1994). It is also unclear how much, if any, of the "β-structure" in pea phytochrome A is contributed by β-sheet vs β-turn, according to CCA analysis (Table 2).

In summary, we have shown that the secondary structure is well conserved between pea and oat phytochromes A. Furthermore, the photoreversible conformational change between P_r and P_{fr} is conserved among these two species as well and might be a general feature of all phytochromes A.

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