Time-Resolved UV Circular Dichroism of Phytochrome A: Folding of the N-Terminal Region

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Variations in a plant's environmental light conditions are sensed by the phytochrome A photoreceptor, which regulates many plant developmental processes.[1-5] This red-absorbing chromoprotein (Pr), like other sensory pigments, is inactive until light absorption initiates a series of dark reactions, leading to formation of the physiologically active far-red-absorbing phytochrome (Pfr). Conformational changes that result from this photoreversible conversion control plant activity. Thus investigations have focused on characterizing the Pr → Pfr mechanism as well as understanding the biological consequences of each step.

Recent circular dichroism (CD) measurements detected a change in ellipticity at 220 nm when Pr was converted to Pfr, indicating that Pr activation results in a 3–5% increase (depending on the buffer system) of α-helix content in the protein.[6-8] Since no CD changes are observed for large phytochrome A (lacking the N-terminus),[8,9] the change in helical structure was localized within the 6-kDa N-terminal domain. Conformational changes of the N-terminal segment are suggested by proteolytic cleavage patterns, phosphorylation sites, and differential antibody binding.[10-12] Additionally, introduction of N-terminal-binding, monoclonal antibodies resulted in suppression of the CD change.[6]

Proteolysis and amino acid substitution experiments have demonstrated that the N-terminal segment is required to stabilize and maintain the chromophore–protein interactions necessary for full biological activity.[13-15] Recent studies by Cherry et al.[13,14] demonstrated that a large phytochrome A mutant transgenically expressed in tobacco had no physiological activity, while wild-type protein produced typical phytochrome A-mediated responses under the same conditions. The mutant phytochrome A lacked biological activity, even though it was expressed and stable at the same levels as the wild type, incorporated chromophore, and was photoreversible. This evidence suggests that the N-terminus of phytochrome A plays a signficant functional role, such as in receptor binding, although direct docking between a receptor and the N-terminus has yet to be identified.

Stable forms of Pr and Pfr are optically distinguishable by their characteristic absorption maxima at 666 and 730 nm, respectively. Although the red shift has been attributed to conformational changes of the tetrapyrrole chromophore pocket and the resulting decrease in coupling between the chromophore and nearby amino acid residues,[16-19] the details of these changes are still unknown. A time-resolved absorption (TROD) study by Zhang et al.[20] identified five intermediates (and lifetimes at 10 °C) during Pr activation: lumi-R1 (7.4 μs), lumi-R2 (89.5 μs), meta-Ra1 (7.6 ms), meta-Ra2 (42.4 ms), and meta-Rc (>266 ms). The important role of the N-terminal conformational changes raises the question of which intermediate is involved in the increased α-helical folding detected in Pfr. TROD and time-resolved circular dichroism (TRCD) studies in the visible region have already associated early lumi-R intermediates with changes in interactions between the chromophore and charged and/or aromatic residues in the chromophore pocket.[17]

To probe the time evolution of the N-terminal α-helix folding, TRCD measurements were performed in the far-UV region. The results reflect enhanced α-helical folding of the N-terminus only during the later stages of phototransformation. The phytochrome A isolation is a modification of the Chai et al.[21] and Vierstra and Quail[9a] preparations. Details of this isolation are described elsewhere.[22] The phytochrome A used in TRCD experiments had a specific absorbance ratio (A_{666}/A_{280}) of approximately 0.9 for the isolated inactive Pr species.

The TRCD technique has already been described in detail.[17,23-26] A Quanta Ray DCR-2 Nd:YAG-pumped dye laser that generates 7-ns (FWHM), 8-mJ pulses at 640 nm was used to photolyze the sample. Transient spectral changes following photoexcitation were monitored with a xenon flashlamp. The spectral resolution was ca. 3 nm, while the time resolution was ca. 900 ns.

The sample was contained in a flow-through cell with two fused silica windows that formed a 2-mm pathlength. A peristaltic pump controlled recycled flow from an iced reservoir to the cell. The Pfr solution was flowed out of the laser beam path after each measurement. During this time, the solutions in the flow-through cell and in the Pr reservoir were irradiated with a high-intensity CW illuminator (Fiber-Lite Series 180, Dolan-Jenner Industries, Inc.). A far-red interference filter (Optometrics Corp.) was used to select 730-nm light for irradiation. These steps served to regenerate Pr from Pfr as well as to introduce inactive Pr into the cell for the next laser flash. At the sample cell, Pr was maintained at 10 °C with a cooling jacket that was connected to a circulating temperature-controlled bath. The integrity and

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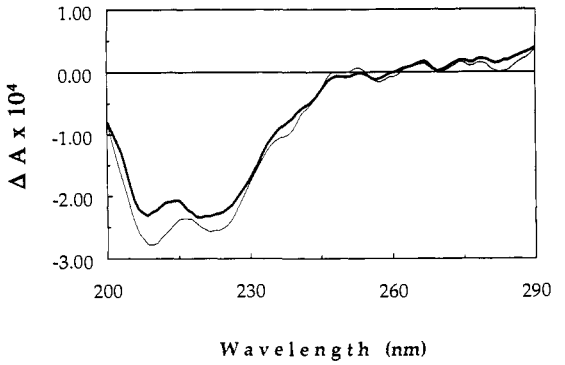

Figure 1. Far-UV–TRCD spectra of Pr *versus* Pfr at 1 s after photoexcitation in 10 mM pH 7.8 Tris buffer with 1 mM EDTA. The Pfr (—) spectrum exhibits an increase in ellipticity at 220 nm relative to the Pr (heavy line) spectrum. These spectra represent 144 averages and are smoothed with a 15-point Savitzky–Golay algorithm. This algorithm has been shown not to distort the spectra,[29] so the spectral resolution remains 3 nm after smoothing.

concentration of the Pr samples were determined by optical spectra measured on an IBM 9420 UV–visible spectrophotometer.

Phototransformation experiments involved TRCD measurements of ground-state Pr and species probed at 100 μs, 20 ms, 100 ms, 500 ms, and 1 s after irradiation of the inactive Pr sample. Data collection was limited by the stability, concentration, and quantity of Pr. Therefore, signal averaging included 14–21 scans (16 averages per scan) of active Pr at each time delay. For 500 ms, only six scans were recorded.

The data used for analyses were difference TRCD spectra, calculated by subtracting the raw TRCD spectrum of pure Pr from that of phototransformed Pr. However, before difference spectra were calculated, the raw Pfr, Pr, and buffer TRCD spectra were offset to 0 from 240 to 260 nm, since this region should have no CD signals. Once offset, the buffer TRCD spectrum was subtracted from the individual Pfr and Pr spectra.

Singular value decomposition (SVD) and global exponential fitting analyses[20,27,28] were performed on the full spectral range of the data. As a method of estimating the uncertainty of the TRCD measurements, data analysis also included the following variations: (1) narrowing the wavelength window to a minimum of 220–225 nm and (2) moving a fixed window of 5 nm across the helical feature at 220 nm. A standard deviation for the exponential fit was calculated from the distribution of lifetimes obtained in these data analyses. Difference TRCD data at all time delays were compiled into a matrix for analysis with routines written in the mathematical software package Matlab (ProMatlab, The Math Works, Inc.).

Figure 1 shows the typical CD spectra of Pr and the species present 1 s after irradiation of Pr in Tris buffer. Use of Tris buffer rather than phosphate buffer results in better light transmission in the far-UV as well as a more distinct change in the Pr and Pfr TRCD spectra. However, most spectra were measured in phosphate buffer so that TRCD changes could be correlated to the Pr → Pfr intermediate decay rates which were determined in phosphate buffer.[20]

The temporal behavior of the α-helical feature was fit to a single exponential with a lifetime of 113 ± 46 ms. A plot of average $\Delta\Delta\epsilon$ *versus* time was generated to show how the changes in ellipticity at 220 nm compare with the exponential fit. Figure 2 compares experimental data in the wavelength range of 210–240 nm to a single exponential curve with a lifetime of 113 ms. The data are also compared to single exponential functions that correspond to the upper and lower limits of 159 and 67 ms.

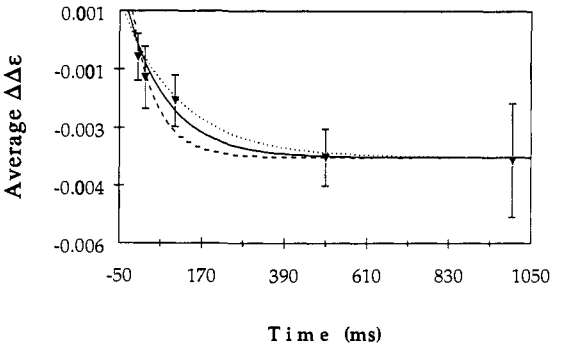

Figure 2. Comparison of the experimental data with single exponential functions calculated according to the equation $\Delta\Delta\epsilon = 0.0034(e^{-t/\tau} - 1)$. The exponential curves have decay rates of 113 (—), 159 (···), and 67 ms (- - -).

SVD and global analyses of the UV–TRCD data associate the enhanced α-helical folding in Pfr with decay of meta-Ra2 and/or meta-Rc. These results are consistent with studies that show retardation of only the last meta-Ra2-to-meta-Rc step upon increasing the solvent microviscosity with glycerol.[30] Closer evaluation of the UV–TRCD results suggest that changes in the α-helical content are more likely due to meta-Ra2 (40 ms) rather than to meta-Rc (>266 ms). Even at the upper limit of 159 ms, the lifetime of the helical change is well below the lower limit of 266 ms for the meta-Rc decay rate.[20]

It is now possible to propose a time course for structural changes that occur upon photoconversion of Pr. The first event detectable in visible TRCD and TROD studies is a chromophore rearrangement confined to the chromophore pocket and attributed to lumi-R intermediates. This step probably reflects the *(Z,Z,Z)*-to-*(Z,Z,E)* tetrapyrrole isomerization, a process that has been gaining experimental support as the primary photoprocess (refs 16,19, 31–35, and references therein). Recent fluorescence energy-transfer studies by Farrens et al.[36] strongly suggest that in Pfr, the chromophore-to-N-terminus distance decreases by ca. 10–12 Å. This UV–TRCD experiment shows that formation of the final Pfr configuration is preceded by at least one detectable protein rearrangement. *(Z)*-to-*(E)* isomerization and subsequent movement of the chromophore may introduce to the surrounding protein a strain that is manifested in part as a helical change.[37] These results, the first nanosecond time-resolved CD measurements of a protein in the far-UV region, demonstrate the value of the UV–TRCD technique to mechanistic studies of protein systems.

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