Time-Resolved Circular Dichroism of Native Oat Phytochrome Photointermediates

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Abstract: Time-resolved circular dichroism (TRCD) spectral studies of the Pr → Pfr phototransformation in native oat phytochrome (124 kDa) were performed in the 500–800 nm spectral region from 500 ns to 500 ms after photolysis at 10 °C. The first intermediate observed in our measurements, lumi-R, had an absorption maximum at 695 nm, but its difference TRCD spectrum showed a CD maximum near 660 nm. This result differed from results of previous steady-state CD measurements where the intermediates of the photoconversion were trapped at low temperatures. Possible explanations for the discrepancies in the TRCD results vs low-temperature CD results are discussed. We conclude that the first species trapped in the low-temperature CD experiment is most probably an unrelaxed lumi-R-type intermediate. The subsequent species probed during the Pr → Pfr phototransformation have similar CD spectra. The structural implications of the TRCD results are discussed.

Introduction

Phytochrome is the photoreceptor in green plants which acts like a photoactivatable switch to regulate physiological processes such as synthesis of chlorophyll, seed germination, induction of flowering, and other photomorphogenetic functions.[1] As a photoreversible photochromic molecule phytochrome exists in two stable forms: the inactive (red-absorbing) Pr and the physiologically active (far-red-absorbing) Pfr forms.[2-4]

$$\mathrm{Pr} \underset{730\ \mathrm{nm}}{\overset{660\ \mathrm{nm}}{\rightleftharpoons}} \mathrm{Pfr} \longrightarrow \text{physiological reactions}$$

The chromophore in Pr is the 15*Z* isomer and the Pfr chromophore the 15*E* isomer of a tetrapyrrole. Thus an isomerization must be involved in the Pr → Pfr transformation.[5-10] Since the chromophore seems to be less reactive in the Pr form than in the Pfr form,[11-13] it has been suggested that in the Pfr form it is located inside a hydrophobic crevice and therefore is more protected from the environment.[11] What structural differences between Pr and Pfr trigger the signal to the plant apparatus remains an important, still unanswered question.[4]

It is generally believed that there are several intermediates in the pathway between Pr and Pfr. There has been a considerable amount of discussion of the transformation of Pr to Pfr, with reports of sequential[3,14] and parallel[1,15-18] pathways. Eilfeld et al. published a simple sequential scheme of the photoinversion:[14]

$$\mathrm{Pr} \xrightarrow[h\nu]{667\ \mathrm{nm}} \text{lumi-R} \xrightarrow{693\ \mathrm{nm}} \text{meta-Ra} \xrightarrow{663\ \mathrm{nm}} \text{meta-Rc} \xrightarrow{725\ \mathrm{nm}} \mathrm{Pfr} \qquad 741\ \mathrm{nm}$$

We performed time-resolved absorption studies on the Pr → Pfr phototransformation from 100 ns to 800 ms after photolysis and found that a mechanism more complicated than the one shown above was needed to describe the transformation.[19] We obtained decay rates for the different intermediates by analyzing the data with a global exponential fitting procedure in conjunction with a singular value decomposition (SVD) method. The results from this analysis indicate that the data is best fit by assuming five kinetic intermediates for the transformation. The lifetimes for these intermediates at 10 °C were found to be 7.4 μs, 89.5 μs, 7.6 ms, 42.4 ms, and at least 266 ms.

The present study was initiated since circular dichroism of a protein-bound chromophore is sensitive to the asymmetry of the chromophore and its surroundings, so that CD should reveal new information about the chromophore and its protein environment. Because the CD of phytochrome in the red/far red spectral region is mostly due to the asymmetrical binding of the chromophore to the apoprotein,[20] the CD spectra should be more sensitive than the absorption spectra to the conformational changes of the chromophore binding pocket during the phototransformation. CD spectra of phytochrome photointermediates trapped at low temperatures have been published recently.[21] However, to obtain the CD spectra of the photointermediates at physiological temperature, the use of time-resolved CD (TRCD) techniques is required. The transient CD spectra obtained at physiological temperature should give a more accurate picture of the photointermediates than those obtained at low temperatures if certain protein motions are inhibited at low temperature. We have developed a technique to measure room-temperature TRCD spectra and kinetics on a nanosecond time scale and have applied this technique to several systems of biophysical interest.[22-28] These

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include the CO recombination reactions of hemoglobin[22-25] and myoglobin[22,23,26] and the investigation of the triplet state of 4-thiouridine in aqueous solutions of tRNA.[27] Although this technique is sensitive to a variety of optical artifacts, we have shown that it is possible to obtain CD data free from photoselectively induced artifacts.[23-25] We applied the TRCD technique to the phototransformation of native phytochrome in the hope that the results would help to reveal the structural changes that the protein undergoes during this transition. We report here TRCD studies of the Pr → Pfr phototransformation from 500 ns to 500 ms after photolysis at 10 °C and compare the results with previously obtained low-temperature CD spectra.[21] We also present a discussion of the mechanistic implications of our results.

Materials and Methods

Native 124 kDa oat phytochrome was isolated from etiolated Garry oat seedlings (*Avena sativa*), according to a modification of the method of Chai et al.[29] Briefly, this entails the harvesting of 3.5 day old etiolated oat seedlings. These seedlings were ground up using a Waring blender, and particulate matter was removed by centrifugation. An ammonium sulfate cut was followed by application of the protein mixture onto a hydroxylapatite column. The column was washed with increasing concentrations of potassium phosphate, finally eluting the phytochrome with 60 mM potassium phosphate. The phytochrome eluted from the column was reprecipitated using ammonium sulfate; the pellet was washed once with 20 mM potassium phosphate buffer (KPB) (1 mL of 20 mM KPB per 2.5 mg of phytochrome) and finally dissolved in the 20 mM KBP (1 mL of KPB per 3 mg of phytochrome). SAR values (666-nm absorbance/280-nm absorbance) of 0.62–0.78 were obtained for these samples. Leupeptin was added to the final phytochrome preparations at a concentration of 10 μg/mL, glycerol was added to a final concentration of 5–10%, and the samples were frozen on dry ice until used for the studies. The absorbance ratio of A_{730}/A_{674} was 1.4 or greater for all samples, ensuring that the phytochrome was undergraded. Approximately 50 mg of phytochrome was used for these studies.

The TRCD technique has been described in detail previously.[22-28] The sample was photolyzed by 7-ns (fwhm), 5-mJ pulses from a Quanta Ray DCR-2 Nd:YAG-pumped dye laser. The actinic phase was at 638 nm with a 10-nm bandwidth due to the contribution of asymmetric spontaneous emission. A xenon flashlamp producing 5-μs pulses was used as the probe light source. The transient CD signals following photolysis were measured by spectral measurements at selected times after excitation and kinetic measurements at selected wavelengths. In the spectral measurements, the broad spectral band of the probe light was dispersed with a Jarrell Ash Monospec-27 spectrograph using a 150 grooves/mm grating and a 500-μm slit. The measurements were made by using a gated optical multichannel analyzer (OMA) detection system (PAR 1420 detector).[30] The OMA sampling gate was delayed jointly with the flashlamp relative to the laser and had a duration of 2 μs when the delay time was 500 ns after photolysis and 8 μs for all other probe times. The ground-state CD spectra of Pr were obtained in the TRCD apparatus while blocking the actinic pulse. The ground-state CD spectrum of Pfr was obtained in a similar way after converting Pr to Pfr by exciting the Pr sample with several actinic pulses. For the kinetic measurements, the transient signals at 660 and 695 nm were detected by an EMI D279 photomultiplier tube mounted on a monochromator (1018B, Precision Instrument, Concord, CA). The signals were collected and digitized by a 500-MHz data acquisition system (7912AD, Tektronix Inc., Beaverton, OR). The slit width of the monochromator was 1500 μm, yielding a spectral resolution of 3 nm, and the impedance of the digitizing oscilloscope was 1 kΩ, 20 pF, yielding a temporal resolution of 20 ns. The

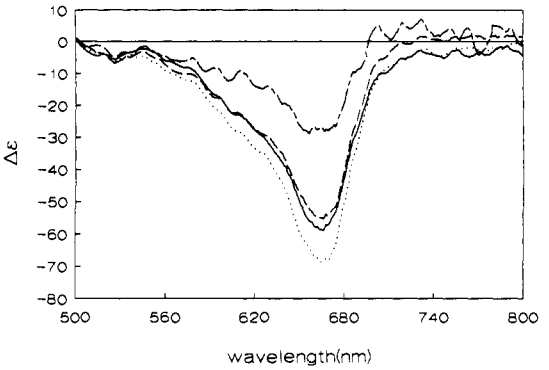

Figure 1. CD spectra of phytochrome measured on the TRCD apparatus. The CD spectra of Pr (···) and Pfr (– – –) represent 448 and 108 averages, respectively. Pfr was obtained by exciting the sample with 5–8 actinic pulses at 638 nm. The TRCD spectra of phytochrome at 500 ns (—) and 500 ms (– – –) after photolysis were obtained with 816 and 456 averages, respectively. The CD spectra of Pr and Pfr and the TRCD spectrum at 500 ms after photolysis were obtained with an 8-μs sampling gate, while the TRCD spectrum at 500 ns after photolysis was obtained using an OMA sampling gate of 2 μs. All measurements were performed at 10 °C.

transient OD signals obtained in the kinetic experiment were measured by inserting a quarter-wave plate between the two polarizers used in the experiment. This greatly increased the detected probe beam intensity, making it possible to reduce the monochromator slit width to 100 μm and eliminate most of the laser scatter.

The 6.1 μM phytochrome sample (OD = 0.8 at 667 nm) was obtained in a 10-mm pathlength, four-window flow cell with the temperature stabilized at 10 °C. Quartz optical windows with negligible strain were mounted on all sides of the flow cell. This cell enabled the actinic beam to enter the sample at a 90° angle relative to the probe beam. A cw light source, entering collinearly with the actinic beam from the opposite window, was used to photochemically regenerate Pr after each laser photolysis. A HOYA R-72 optical filter was placed between the sample and this cw lamp so that the illumination wavelength was greater than 730 nm. The sample was illuminated for 5–8 s between each photolysis pulse. The illumination period was extensively tested to ensure the complete conversion of the photolyzed sample back to Pr. This was demonstrated by the fact that the absorption spectrum of the sample after the illumination was the same as that before the photolysis pulse. The experiment was performed in sets of 8 averages.

As has been described previously,[22,24,25] in the TRCD technique elliptical light is produced by introducing a strained (1°) fused silica plate in the path of a linearly polarized probe beam. The strain axis is set at +45° or −45° relative to the polarization axis of the incident light, producing right elliptically (REP) or left elliptically (LEP) polarized light, respectively. A circularly dichroic sample will affect the relative ellipticities of REP and LEP. This change in ellipticity is determined by measuring the change in intensity along the direction of the minor ellipse axis. This is accomplished by passing the light through a second polarizer oriented perpendicular to the original polarization axis. The CD signal is calculated by

$$S = (I_R - I_L)/(I_R + I_L)$$

where I_R and I_L are the detected intensities when REP or LEP is incident on the sample. The signal is related to $\Delta\epsilon$ by[22,24,25]

$$\Delta\epsilon = S\delta/Cz$$

where δ is the retardation of the strain plate in radians, C is the sample concentration in moles/liter, and z is the path length in centimeters. The CD data presented here was smoothed according to the least-square procedure of Savitzky and Golay,[31] using 25 points to yield optimal smoothing consistent with our spectral resolution.

The transient *difference* CD signals ($\Delta\Delta\epsilon$) are simply the CD measured at a specific delay time after photolysis minus the unphotolyzed Pr CD. Transient difference OD spectra (ΔOD) are calculated using the I_R and I_L intensities from the TRCD measurements, according to

$$\Delta\text{OD} = -\log\left((I_R + I_L)_t/(I_R + I_L)_{t=0}\right)$$

where the numerator is the average of the REP and LEP light intensities for the intermediate probed at time t, and the denominator is the cor-

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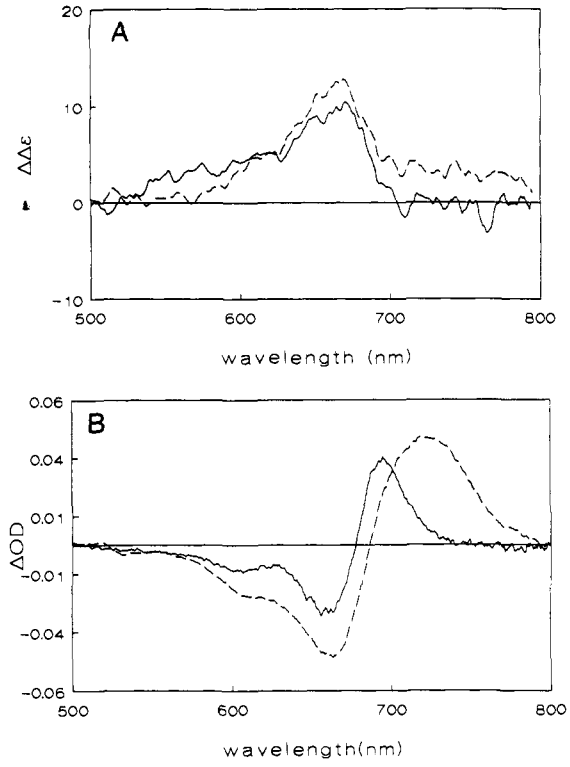

Figure 2. (A) *Difference* CD spectra of the TRCD data shown in Figure 1. The transient *difference* CD spectra of phytochrome at 500 ns (—) and 500 ms (---) after photolysis were obtained by subtracting the CD spectrum of Pr from the TRCD spectra at those delay times. To obtain the absolute spectra of the species present at these times, one would have to add the CD spectrum of unphotolyzed Pr to the difference spectra, as discussed in the text. All of the data were smoothed by the Savitzky–Golay algorithm using 25 points. (B) Calculated *difference* OD spectra (see Materials and Methods) from the TRCD data at 500 ns (—) and 500 ms (---) after the photolysis of Pr.

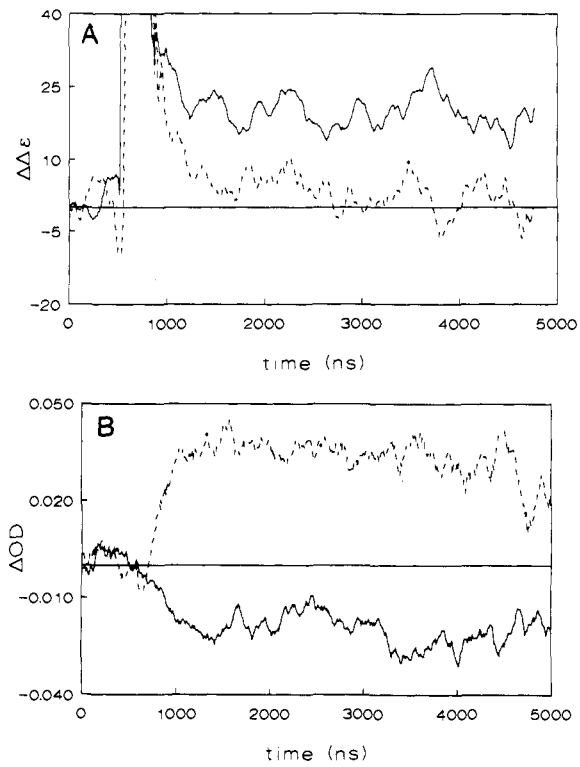

Figure 3. (A) *Difference* kinetic CD signals following the photolysis of Pr. The transient *difference* CD signals of phytochrome monitored at 660 (—) and 695 nm (---) represent 220 and 160 averages, respectively. These difference signals were obtained by subtracting the CD before photolysis (i.e., CD of Pr) from that after photolysis and were smoothed by the Savitzky–Golay algorithm using 25 points. Overload of the detection system due to laser scatter and sample emission obscures the first 1 μs of the TRCD signal. (B) *Difference* kinetic OD signals monitored at 660 (—) and 695 nm (---) following the photolysis of Pr. These transient signals are obtained by subtracting the prelaser OD (i.e., OD of Pr) from that after laser photolysis and represent only one average.

responding average intensities for the unphotolyzed Pr sample. The difference OD spectra are calculated to verify the formation of the species of interest and might not exactly correspond to published absorption spectra due to base line drifts in the TRCD experiment.

Results

Figure 1 shows the Pr and Pfr CD spectra and the TRCD spectra obtained at 500 ns and 500 ms after photolysis of Pr at 10 °C. In order to ensure the detection of the lumi-R species, the first delay time was set to be 500 ns after photolysis. The OMA sampling gate was set to be 2 μs for this measurement, so that intermediates appearing from 0.5 to 2.5 μs after photolysis were detected. This earliest probe time was chosen to occur well after the molecular reorientation time of 103.5 ns[32] so as to avoid photoselectively induced artifacts.[24,25] The curves are offset to give zero signal around 500 nm. Figure 2A,B shows the transient *difference* CD and OD spectra, respectively, calculated from the data shown in Figure 1. These spectra are also corrected for zero signal around 500 nm. To obtain the absolute CD spectra of the species present at each probe time, one would have to add an appropriate amount of the CD spectrum of unphotolyzed Pr to the difference spectra. The CD intensity of Pr is large relative to the CD intensity of the species generated after photolysis, so that the shape of the CD spectra calculated by adding back the Pr spectrum is very sensitive to the photolysis yield in the experiment. Since we do not have an accurate measure of the photolysis yield, the absolute spectra of the species present at 500 ns and 500 ms are not presented. It is clear, however, that the *difference* CD at 695 nm is negligible in comparison to that at 660 nm for the 500-ns species. This result is reproducible in all of the TRCD experiments and is surprising in light of previous low-temperature CD studies which suggest that the CD spectrum of lumi-R has a maximum at 695 nm.[21]

To investigate this further and to assure that this result was not due to artifacts such as photoselectively induced linear birefringence artifacts, we performed kinetic CD measurements at 660 and 695 nm from 0 to 5 μs after photolysis. The results of this experiment are shown in Figure 3A. Figure 3B shows kinetic OD spectra at the same wavelengths measured on the TRCD apparatus in the same experiment. This is shown to verify that the sample exhibited the proper absorption difference spectrum. The data in Figure 3A are set to zero before photolysis, so that the changes in the CD signal following photolysis can be compared more directly. Overload of the detection system due to laser scatter and sample emission obscures the first 1 μs of the TRCD signal. This is mainly caused by the use of large slit width (1500 μm) and impedance (1 kΩ) in the kinetic measurements to yield sufficient detection sensitivity. This was made necessary by the very low light levels due to the low intensity of the probe light in the red spectral region, as well as to the nature of the TRCD technique. The TROD data was not plagued with laser scatter because it was possible to make these measurements with much smaller slit widths than with the TRCD data.

Figure 4 shows the transient *difference* CD and OD spectra at times ranging from 10 μs to 10 ms after photolysis. The data in this figure represent far fewer averages than that in Figure 2 and are therefore noisier. The difference OD spectra in Figure 4B indicate that there is a significantly higher photolysis yield in that experiment than in the experiment shown in Figure 2. The ΔOD signals obtained at 10 μs and 10 ms should be considerably smaller than those at 500 ns and 500 ms, respectively,[19] but both the 10 μs vs 500 ns and the 10 ms vs 500 ms signals have similar magnitudes (Figures 2B and 4B). Consequently, the difference

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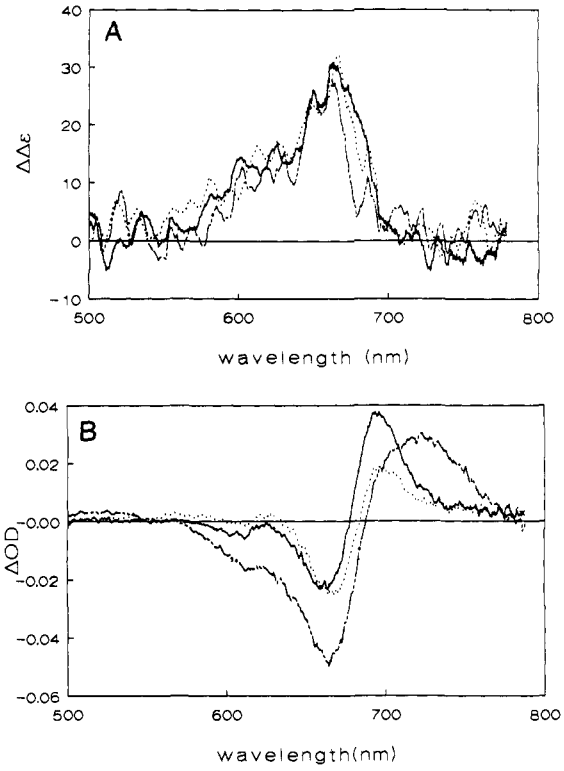

Figure 4. (A) *Difference* CD spectra at 10 μs (—), 100 μs (···), and 10 ms (- - -) after the photolysis of Pr. These CD spectra were measured on the TRCD apparatus with a 5–8-μs OMA gate. All of the data represent 244 averages, smoothed by the Savitzky–Golay algorithm using 25 points. (B) Calculated *difference* OD spectra (see Materials and Methods) of the data presented in A. These OD spectra were calculated from the TRCD data at 10 μs (—), 100 μs (···), and 10 ms (- - -) after photolysis, respectively.

CD spectra in Figure 4A are larger than those shown in Figure 2A. Another reason for the discrepancies in the relative sizes of the difference CD spectra in Figures 2A and 4A is that the extinction of the polarizers is better in the measurements illustrated in Figure 4, resulting in larger CD signals.[24,25] The TRCD data depicted in Figure 3A indicate that the CD change at 660 nm for the lumi-R intermediate is comparable in size to the CD changes shown in Figure 4, and therefore the sizes of the CD of lumi-R and the later intermediates are similar. This is also clear from the fact that the CD signal in Figure 3A does not change significantly during 5 μs even though the lifetime of lumi-R is found to be 7 μs. However, whenever data involved in such a comparison are obtained from different experiments, the comparison is only qualitative since it depends strongly on experimental conditions, as mentioned above.

Discussion

TRCD of lumi-R and Pfr. The difference OD spectrum shown in Figure 2B clearly indicates that the species probed between 500 ns and 2.5 μs after photolysis in the TRCD experiment is the lumi-R intermediate. Global analysis of TROD data obtained in our laboratory indicates that the first detectable intermediate has a decay time of 7.4 μs at 10 °C and a difference absorption spectrum identical to the one shown here.[19] A notable feature of the *difference* CD spectrum at 500 ns following photolysis (Figure 2A) is that the maximum is around 660 nm, while the difference absorption maximum is around 695 nm (see Figure 2B). We consistently observe that the difference CD spectrum at 695 nm is smaller than that at 660 nm for the lumi-R intermediate, as shown in Figure 2A. This result is supported by the kinetic data shown in Figure 3A in which the CD changes at 660 nm are considerably larger than the very small changes at 695 nm. As shown in Figure 3A, the signals are constant within the signal/noise ratio for the duration of the experiment (5 μs). Figure 3B depicts the OD changes at 660 and 695 nm which, within the signal/noise ratio, are in excellent agreement with the difference OD spectrum of the 500-ns species in Figure 2B, showing that the same species is being probed in the kinetic and spectral experiments. Another reproducible result observed in the difference CD spectrum of the lumi-R species is the shoulder in the blue part of the spectrum around 550 nm. This result is quite surprising since it is not in the immediate vicinity of the known absorption maxima of the lumi-R species. The implication of this result will be discussed in more detail below. The difference absorption spectrum at 500 ms after photolysis (Figure 2B) indicates that the difference CD spectrum measured at this time (Figure 2A) should represent that of Pfr. This is in agreement with our TROD results in that the rise time of Pfr is approximately 266 ms at 10 °C.[19] The fact that this difference CD spectrum exhibits the red shoulder around 730 nm is consistent with the difference CD spectrum of Pfr obtained by steady-state measurements.[21]

The TRCD data for the lumi-R photointermediate shown in Figures 1 and 2A exhibit marked differences from the steady-state low-temperature CD spectra found in the literature.[21] Eilfeld and Eilfeld published CD and difference absorption spectra of a species trapped at −110 °C and assigned the trapped species to lumi-R. The absorption difference spectrum they obtained closely resembles the TROD spectrum at 500 ns following photolysis shown in Figure 2B, while the difference CD spectrum shows a maximum at 695 nm with a broad blue shoulder extending to around 570 nm.[21] This spectrum looks similar in shape to the TRCD spectrum at 500 ns after photolysis shown in Figure 2A, but it is red-shifted by about 30 nm. The good agreement found between the TRCD at 500 ms after photolysis (see Figure 2A) and the steady-state CD spectra of Pfr[21] indicates that our TRCD apparatus is capable of measuring CD signals in the 700–800 nm spectral region. Therefore, the lack of difference TRCD signal at 695 nm in the lumi-R intermediate cannot be due to the limitation of the experimental setup.

Potential Experimental Artifacts in Low-Temperature CD and TRCD Measurements. Several experimental factors could potentially contribute to the differences in the CD spectra of the lumi-R intermediate measured by the TRCD technique and the previous low-temperature study. The first possibility is that the CD spectrum of lumi-R is temperature dependent and undergoes a red shift as the temperature is decreased to about −110 °C, at which the low-temperature CD spectrum of lumi-R is obtained, while the absorption spectrum is much less sensitive to temperature changes and stays constant when the temperature is lowered. Burke and Pratt[33] found a large temperature dependence of the CD spectra for both Pr and Pfr of small oat phytochrome, while the absorption spectra of these species were quite insensitive to the same temperature changes. Eilfeld and Eilfeld also mention that the CD spectrum of Pr changes slightly with temperature.[21] Another possibility is that the CD spectrum obtained by Eilfeld and Eilfeld contains artifacts due to low-temperature effects. They mentioned that the buffer–glycerol system "exhibited pronounced polarization effects" without showing crystallizations or cracks at temperatures below −110 °C. They also noticed that the CD bands of Pr at −110 °C were broadened and significantly less intense than those at room temperature and suggested that this was largely due to depolarization effects.[21]

A third reason for the observed differences could be that the results of the TRCD technique are due to photoselectively induced linear artifacts, such as linear birefringence (LB) and linear dichroism (LD). As was discussed in the Materials and Methods section, the delay time for probing the lumi-R intermediate was chosen to be significantly longer than the rotational time of phytochrome since both LB and LD artifacts disappeared after the sample underwent molecular reorientation after light excitation. Thus, such artifacts cannot contribute to our CD spectrum of lumi-R obtained between 500 and 2500 ns. The shoulder in the blue part of the difference CD spectrum of lumi-R (Figure 2A) might also be suspected of being due to LB, since it is not

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in the vicinity of the lumi-R absorption maxima. While CD is an absorptive property of the chromophore, LB is a dispersive property and can thus occur outside an absorption band. However, the $\Delta\epsilon/\epsilon$ value for the 550-nm region in the 500-ns TRCD spectrum is on the order of 10^{-3}, typical for CD bands.[22] LB usually yields a broad asymmetrical signal shape centered around the absorption maxima, as we have illustrated previously.[24,25] This means that, if the shoulder around 570 nm were due to LB, there would most likely be LB contributing to the CD signal around the peaks of the absorption bands (660 and 695 nm) as well. In addition, the kinetic CD data obtained at 660 and 695 nm (see Figure 3A) does not decay within 5 μs, which is much longer than the rotational time of the protein.[32] A decay would be expected if photoselectively induced linear artifacts gave rise to the transient signals since these artifacts disappear after the sample undergoes orientational randomization. Thus the results obtained in this TRCD study cannot be explained by photoselection artifacts. The blue shoulder in the TRCD difference spectrum of lumi-R must be a forbidden transition which has very small oscillator strength and a relatively large rotational strength.

Another possible explanation for the discrepancies between the two different CD studies would be that in the low-temperature experiment prelumi-R was actually trapped, so that the CD spectrum presented was not of lumi-R but of prelumi-R. There is some evidence that lumi-R is formed from a prior intermediate, prelumi-R, rather than directly from the excited state of Pr.[34-37] Song et al. performed absorption studies of large phytochrome at 77 K and found a photoreversible intermediate with an absorption maximum at 694–696 nm, which was assigned a prelumi-R.[34] Fluorescence experiments on native phytochrome performed by the same laboratory indicated that the excited singlet state of Pr decayed to a primary intermediate, which then decayed into lumi-R.[35,36] From picosecond absorption and fluorescence studies Lippitsch et al. also found evidence that lumi-R was not directly formed from the S_1 state but must have had a precursor which they called prelumi-R. This conclusion is derived from absorption and fluorescence results which show that the S_1 population relaxes within 35–45 ps. A stationary lumi-R absorption at 690 nm appears with a delay of 35 ps and a rise time of 60 ps, but is completely established only at 150 ps after excitation. The authors propose that prelumi-R is formed from the S_1 state within 35 ps and decays within 60 ps into lumi-R. However, they do not report an absorption maximum for prelumi-R.[37] If prelumi-R, instead of lumi-R, were trapped at −100 °C, the low-temperature absorption maxima of prelumi-R and lumi-R would have to be the same, as reported by Song,[34] but the CD spectra would have to be different. By close inspection of the picosecond absorption spectra published by Lippitsch et al.,[37] it is evident that the difference absorption spectrum of prelumi-R at 35 ps after photolysis does not have a maximum near 695 nm at room temperature. However, the picosecond absorption data is not very clear in this region of the spectrum. More picosecond absorption studies of phytochrome are clearly needed in order to support or discredit this hypothesis.

The possibilities discussed above are presented as conceivable explanations for the differences observed between the low-temperature CD and TRCD experiments. We would like to emphasize, however, that we do not believe that any of these alternatives are viable. We discuss them here to demonstrate that several possible explanations for the results obtained have been considered. A discussion of the most likely reason for the differences observed is presented below.

Origins of Phytochrome Absorption and CD. There are two visible absorption bands of Pr probed in the TRCD experiment, Q_y, centered around 668 nm, and Q_x, around 608 nm.[38] It is necessary to emphasize, however, that the phytochrome chromophore is significantly different from the porphyrin chromophore found in heme proteins where this terminology is more widely used. From the absorption and CD spectra it is clear that the Q_y transition has higher oscillator and rotational strength than the Q_x transition. In lumi-R, the absorption spectrum indicates that the Q_y transition must be centered around 695 nm and Q_x around 660 nm. Analogous to Pr, the Q_y transition has a higher oscillator strength than Q_x in lumi-R (see Figure 2B). However, the rotational strength of Q_y must decrease while the Q_x rotational strength increases or remains constant during the phototransformation from Pr. The fact that the maxima of the absorption and CD spectra do not overlap in the lumi-R intermediate (see Figure 2) clearly illustrates the differences in the sensitivities of absorption and circular dichroism to the molecular structure.

The features of the absorption spectra of Pr, Pfr, and the photointermediates are determined by the conformation of the tetrapyrrole chromophore and the protein–chromophore interaction. Sugimoto and co-workers propose a model for the phytochrome binding pocket which contains a protonated tetrapyrrole (proposed at the nitrogen of the pyrrole ring C), a negative point charge, and a point dipole.[39] Their molecular orbital calculations demonstrated that the changes in the absorption maxima of Pr, Pfr, and the intermediates could be explained by the movements of the negative charge and the dipole relative to the protonated chromophore. They found that the "ionic structure" of the chromophore binding pocket is necessary in order to predict the absorption spectra of the intermediates and Pfr.[40,41] This negative charge could be a glutamate or an aspartate amino acid in close proximity to the chromophore.

This simple model is supported by some experimental evidence. It has been proposed that the primary photochemical reaction of phytochrome after light excitation is chromophore-localized and most probably a $Z \rightarrow E$ isomerization.[42,43] The red shift of the absorption maximum of lumi-R could be predicted by the increase of the distance between the protonated chromophore and negatively charged amino acid(s) due to the isomerization. Rüdiger and co-workers[8] suggest that the lumi-R to meta-Ra transformation is accompanied by the deprotonation of the chromophore, which leads to the blue shift of the absorption spectrum of meta-Ra relative to that of lumi-R. This suggestion is supported by the resonance Raman results of Tokutomi and co-workers, who found that the protonation state of one of the chromophore rings (ring C) is different for Pr and Pfr in large pea phytochrome.[44,45] Thümmler and Rüdiger[46] argue that the large bathochromic shift in the absorption band of Pfr cannot be directly connected with the chromophore configuration, but must involve protein–chromophore interactions. They propose that this large bathochromic shift is due to the presence of a positively charged amino acid near the chromophore.[8,47] Therefore, it is reasonable to postulate that the absorption spectra of Pr, Pfr, and the photointermediates are largely determined by the protonation status of the tetrapyrrole and the interaction between the chromophore and the nearby charged amino acid residue(s).

The CD of a protein-bound chromophore is often due at least in part to asymmetrical electric dipole interactions with nearby

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aromatic amino acids in the binding pocket. The CD of the chromophore absorption band could also arise from the asymmetrical distortion of the chromophore conformation from that of a free chromophore in solution due to protein constraints. Although it is not a trivial matter to determine which mechanism contributes most significantly to the observed CD of the chromophore absorption band, experimental and theoretical CD studies on heme proteins[48,49] and visual pigments[50,51] indicate that the asymmetrical dipole interactions between the bound chromophore and the surrounding amino acids contribute predominantly to the chromophore CD.

In myoglobin and hemoglobin, for example, there are no indications that the achiral heme chromophore loses its planarity when it is situated inside the globin so as to make it asymmetrical and give rise to the observed heme CD bands. Hsu and Woody calculated the rotational strength of interactions between the heme chromophore and side chains of the surrounding globin according to the extended Kirkwood theory.[52] They found that the coupled oscillator interaction between the heme transitions and allowed $\pi-\pi^*$ transitions of nearby aromatic amino acid chains could account for the observed Soret CD band. Hsu and Woody calculated the contribution to the Soret rotational strength from $\sigma-\sigma^*$ transitions of alkyl side chains and from $n-\pi^*$ and $\pi-\pi^*$ transitions of the polypeptide backbone as well. However, they found that the contributions from these transitions were orders of magnitude smaller than those from the aromatic amino acids.[48,49]

Kropf and co-workers[50] have proposed that for rhodopsin the CD of the main 11-*cis*-retinylidene chromophore absorption band (~500 nm) and the cis band (~350 nm) was mainly due to the interaction between the 11-cis chromophore and the indole side chain of a nearby tryptophan. They use this mechanism to explain the fact that, while the absorption intensity of the cis band is only about 10% of the main band, the CD of the cis band is larger than that of the main band. This phenomenon is likely due to the fact that the rotational strength derived from the asymmetrical dipole interactions between two chromophores is proportional to $D_1D_2\lambda_1\lambda_2/R_{12}^2(\lambda_1^2 - \lambda_2^2)$, where D_1 and D_2 are the dipole strengths, λ_1 and λ_2 are the wavelengths associated with the absorption maxima of the two chromophores, and R_{12} is the distance between the two chromophores. Because the absorption band of an aromatic side chain (~280 nm) has much better overlap with that of the cis band compared to the main band, the dipole–dipole interaction would contribute much more significantly to the CD of the cis band.

Similar arguments could be used to explain the absorption and CD bands of phytochrome. While the rotational strength of the Soret band of Pr (~380 nm) is about three times as large as that of the Q-bands,[21] the oscillator strengths of these two transitions are approximately the same.[3] If there is an aromatic amino acid residue is close proximity to the bound chromophore, the dipole–dipole interaction between an aromatic side chain and the tetrapyrrole will significantly enhance the CD of the Soret band relative to the Q-band of Pr due to the more effective overlap of the absorption bands. In fact, the "overlap factor" (i.e., $\lambda_1\lambda_2/(\lambda_1^2 - \lambda_2^2)$) of the Soret band would contribute about 3 times as much to the rotational strength as the Q-band and thus could qualitatively explain the large CD of the Soret band relative to the Q-band in Pr. Therefore, it seems plausible to assume that the CD of the Q-band in phytochrome is predominantly due to the dipole–dipole interaction of the tetrapyrrole and the adjacent amino acid residue(s).

Since the absorption and circular dichroism of the chromophore in phytochrome are due to different kinds of interactions between the tetrapyrrole chromophore and apoprotein environment, it is not surprising that they convey different spectral changes. As we have discussed above, the different absorption spectra of Pr, Pfr, and the photointermediates are determined to a large extent by the locations of the charged amino acid residues relative to the chromophores. The rather intense CD of the Soret and Q-bands observed in Pr suggests that the chromopore is also in close proximity to one or more aromatic residues. Such a proximity relationship might not drastically change in the lumi-R intermediate produced at low temperature (−110 °C) if the protein is trapped in a conformation which does not allow large change in the tertiary structure of the chromophore pocket. The relatively large CD band at 695 nm in lumi-R observed at −110 °C[21] indicates that the dipole interaction between the chromophore and aromatic residue(s) is still significant after the photoisomerization. As the temperature increases and conformational changes become possible, the chromophore binding pocket would undergo a conformational relaxation to accommodate the new chromophore orientation. This could cause the aromatic residue(s) to move away from the chromophore, as suggested by the decreasing CD in the absorption bands of the later species.[21]

The fact that the CD band at 695 nm is not observed for lumi-R at physiological temperatures (10 °C) implies that a partial protein rearrangement occurs at an early time in the phototransformation. The relaxation of the chromophore and the movement of aromatic residue(s) away from the chromophore must be completed in 1 μs, as suggested by the lack of CD at the 695-nm lumi-R absorption band (Figures 2A and 3A). The charged group(s) near the chromophore, however, is unlikely to undergo any dramatic movement in this early relaxation step, since such movement is expected to cause marked changes in the absorption spectrum of lumi-R, while the transient absorption spectrum of lumi-R (Figures 2B and 3B) is very similar to the low-temperature spectrum. Furthermore, it is unlikely that the CD band at 695 nm in the low-temperature lumi-R spectrum could be due to a distorted chromophore because a distorted chromophore (at low temperature) is also expected to exhibit a different absorption spectrum compared to the transient spectrum of a relaxed chromophore (at higher temperature).

Difference between Low- and High-Temperature lumi-R. From the above arguments we propose that the lumi-R species observed at low temperature is different from that observed at physiological temperature. The earlier conformational relaxation process associated with the aromatic residues in the chromophore binding pocket must occur faster than 1 μs since it escapes our detection in the TRCD experiments. The trapping of an unrelaxed pigment at low temperature is plausible. The $Z \rightarrow E$ isomerization of the tetrapyrrole triggered by light absorption would produce a strained pigment in which the chromophore is in close interaction with the surrounding residues in the binding pocket. This strained pigment, however, might not undergo conformational relaxation at low temperatures (e.g., −110 °C) due to the presence of an activation barrier for the structural rearrangement of the chromophore binding pocket in response to the isomerization. One question is whether the relaxed lumi-R, as detected by TRCD experiments at physiological temperatures, could be trapped at a higher temperature (between the temperature at which the strained lumi-R is observed and the temperature of the lumi-R → meta-Ra transition). This seems unlikely since the temperature at which the CD spectrum of the strained lumi-R was measured (−110 °C) was rather close to the transition temperature of lumi-R to meta-Ra (ca. −100 °C).[3] This in turn raises an interesting question regarding the relationships between the photointermediates observed in low-temperature studies and the corresponding kinetic intermediates by time-resolved spectroscopic measurements at physiological temperature.

The fact that a kinetic photointermediate could not be trapped in a photoreaction at low temperature has been found previously for bovine rhodopsin.[53] Rhodopsin (λ_{max} = 490 nm) is a protein which undergoes 11-cis to all-trans isomerization of its retinal chromophore upon light absorption. An intermediate, bathorhodopsin (BATHO), can be trapped at liquid nitrogen temper-

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ature (77 K). This intermediate exhibits a red shift in the absorption spectrum (λ_{max} = 543 nm) which is partially attributed to a strained chromophore conformation. As the temperature is raised above −140 °C, BATHO undergoes a thermal transition to the lumirhodopsin (LUMI) intermediate, which has a relaxed chromophore conformation and an absorption spectrum similar to that of unphotolyzed rhodopsin. However, Hug et al. observed from time-resolved absorption studies that an additional, blue-shifted intermediate (BSI, λ_{max} = 440 nm) exists between the BATHO and LUMI intermediates.[53] It is found that the BSI intermediate has a larger enthalpy (by ~3 kcal/mol) and a larger entropy (by ~45.1 J/mol·K) than BATHO and is in thermal equilibrium with batho at physiological temperatures. The equilibrium temperature (T_{eq}) of the two intermediates is easily obtained from $\Delta G = \Delta H - T\Delta S = 0$, where ΔG, ΔH, and ΔS represent the differences of free energy, enthalpy, and entropy between BATHO and BSI, respectively. Hence, $T_{eq} = \Delta H/\Delta S \approx 280$ K. It is obvious that bsi is thermodynamically unfavorable at lower temperatures since it is higher in free energy than batho at $T < T_{eq}$. Therefore, while bsi can be observed by time-resolved measurements at physiological temperatures, it cannot be trapped at a low temperature since the equilibrium temperature for BATHO ↔ BSI is higher than that for the BATHO to LUMI transition. This example illustrates the fact that, in general, kinetic intermediates are not guaranteed to have a one-to-one correspondence with low-temperature intermediates. This is a result of the fact that the kinetic pathway of a photoreaction is determined by the activation barriers of the reactions, while the sequence of steady-state intermediates in thermal equilibria observed at low temperatures is primarily determined by the relative values of the equilibrium temperatures ($\Delta H_i/\Delta S_i$).

A similar argument can be used to explain the differences in the lumi-R species observed in the low-temperature steady-state measurements and in our TRCD experiments. This requires that the enthalpy and entropy of lumi-R are higher in the relaxed species than in the strained species, as found for BSI and BATHO. As a result, the strained lumi-R intermediate could be thermodynamically more stable at low temperatures due to the lower enthalpy compared to the relaxed species. However, the equilibrium temperature for the transition between the strained and relaxed lumi-R is higher than the transition temperature for the strained lumi-R to meta-Ra intermediate, so that the relaxed lumi-R would not be observed as a stable intermediate by the low-temperature spectroscopic studies. At physiological temperatures (e.g., 10 °C), the relaxed lumi-R species is lower in free energy due to a more significant contribution of the entropy factor and is thus more thermodynamically favorable compared to the strained species. Consequently, the relaxed lumi-R species is only observed as a transient intermediate in the time-resolved spectroscopic studies performed at higher temperatures. This intermediate, however, could not be effectively distinguished by ordinary absorption experiments due to the similar absorption spectra of the relaxed and the strained species. On the other hand, the partial relaxation of the chromophore binding pocket, which increases the relative distance between the nearby aromatic residue(s) and the chromophore in the early stage of the phototransformation, gives rise to a dramatically reduced CD in the chromophore absorption band of the relaxed lumi-R species compared to that of the strained species. This causes a marked difference between the CD spectrum observed at physiological temperature and that observed at low temperature.

The differences between unrelaxed and relaxed lumi-R must lie in the apoprotein near the chromophore and not at any exposed sites of the protein, since there are reports that the first two steps of the Pr transformation are quite independent of macroscopic environmental conditions.[16,54,55] Ruzsicska et al. found a small temperature dependence of the lifetimes of the primary photoreactions when comparing small and native phytochrome in a flash photolysis study.[54] Krieg et al. performed laser photolysis studies on native phytochrome and found an insensitivity of the lifetimes of the first two intermediates (called I^i_{700} and I^i_{bl}) to the binding of Pr to large unimolecular vesicles composed of different lipids of varying rigidity.[55] Eilfeld et al. performed laser flash photolysis studies of native oat phytochrome in solvents of different microviscosities.[16] They found that the formations of meta-Rc and Pfr were significantly retarded by increasing the microviscosity of the solvent by adding glycerol. The decay of meta-Ra, however, was not retarded under similar circumstances. They conclude that the meta-Ra → meta-Rc process is split into at least two consecutive steps: meta-Ra → meta-Rac → meta-Rc, of which only the latter is strongly retarded in glycerol. The rate constants of these transitions were only slightly affected by adding polydextrane, a high molecular weight compound exhibiting about the same macroscopic viscosity as glycerol but which cannot penetrate the protein as easily as glycerol. From these results the authors propose that the global conformational changes of the protein occur only in the last stage of the phototransformation, i.e., during the meta-Rc → Pfr transition.[16] However, it has recently been suggested that the picosecond relaxation processes of the excited Pr species are not isolated to the chromophore but rather are controlled by the protein moiety, which affects the interactions between the chromophore and the protein. This proposal stems from the picosecond fluorescence studies on phytochrome chromopeptides of different sizes and on deuterated and undeuterated phytochrome, in which the fluorescence decay of these species differs from each other.[56a,b] Surface-enhanced resonance Raman studies indicate that the primary photoprocess may involve the breaking of a hydrogen bond to the chromophore.[57] These results support our suggestion that there is a change in the chromophore–apoprotein interaction during the formation of the lumi-R intermediate at physiological temperatures and that this change is inhibited at −110 °C.

Eilfeld and Eilfeld propose that an isomerization occurs in the initial step of the phototransformation since the CD of the first species trapped at low temperature is dramatically different from that of Pr. They suggest that the initial step of the transition is mainly chromophore localized and that only the subsequent dark relaxations involve conformational changes of the protein microenvironment. They also speculate that the small CD changes found in the meta-Ra to Pfr pathway might imply that the helical structure of the apoprotein in the vicinity of the chromophore does not change much in these later reactions.[21]

It is clear from Figure 1 that Pfr has a small CD. It is thus reasonable to speculate that by the completion of the phototransformation the chromophore pocket undergoes a large change and the amino acids in the chromophore vicinity can no longer couple with the chromophore as well as in the Pr species. It is suggested that the overall chromophore conformations are similar in Pr and Pfr, but that the exocyclic dihedral angles of chromophore in Pfr are twisted by 20° relative to that of Pr.[9] This twist might also reduce the interaction between the chromophore and the apoprotein. It is suggested that the intermediate immediately preceding Pfr in the phototransformation involves a chromophore without strong interaction with the protein.[46] It is conceivable that similar weak chromophore–protein interactions also exist in Pfr.

TRCD of Late Photointermediates. Results from TRCD experiments performed at 10 μs to 10 ms after photolysis are shown in Figure 4A. The TRCD spectra at these times should represent that of later intermediates, as implied by the results of our time-resolved absorption studies at 10 °C.[19] The data presented in this figure involve a significantly smaller number of averages than the data shown in Figure 2A and are thus noisier. Although

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it is difficult to draw any quantitative conclusions from this data, one can still see from the features of the difference spectra that there are no large changes in the signal sizes for the later intermediates. This is consistent with the proposal that there are no significant changes of the overall chromophore conformation in the later stages of the phototransformation[58] or of the helical structure of the chromophore.[21] The data shown in Figure 4A also suggest that the aromatic residues in the chromophore binding pocket are rather far from the chromophore; otherwise the interactions between these residues and the chromophore would give rise to observable CD in the chromophore absorption band. This is consistent with previous phosphorescence results by Sarkar et al.[59] The drastically different absorption spectra of these intermediates (see Figure 4B) must be due to the protonation of the chromophore and/or to significant changes in the positions of the surrounding charged residues relative to be chromophore. This would explain the large red-shifted absorption and the small CD band of Pfr, since the movement of these charged residues might not couple effectively with the chromophore to significantly affect the CD, while they could dramatically change the absorption spectrum of the pigment. The conformational changes in the later stages of the phototransformation might occur in the more exposed regions of the protein, as discussed above. These changes would be detected if one observed the CD changes in the UV part of the spectrum where the aromatic amino acids absorb. Work is currently underway in this laboratory to extend the TRCD technique into that part of the spectrum.

The TRCD data presented in Figure 4A is quite consistent with the low-temperature steady-state results obtained by Eilfeld and Eilfeld in which the later intermediates are trapped sequentially as the temperature is raised.[21] However, their results show slightly larger CD changes between the different intermediates. One reason for the smaller variations in the difference TRCD spectra of the later intermediates could be that at ambient temperatures a photointermediate has a higher probability of dark reversion back to Pr than at low temperatures. Eilfeld and Eilfeld mention that at temperatures above 4 °C more than 70% of the quantum yield for the photoprocess may be lost by dark reversion reactions upon transformation of lumi-R to Pfr, while such reversions seem to be smaller or even nonexistent at low temperatures.[21] This would explain why the difference CD spectra of the later intermediates are larger in the low-temperature experiment than what is shown here. This effect would also be seen in the corresponding TROD data, but the absorption spectra of the different experiments could not be compared since Eilfeld and Eilfeld did not publish the absorption spectra for the later intermediates. A better signal/noise ratio is clearly needed to draw further conclusions for the data presented in Figure 4.

Conclusions

We have presented TRCD spectra of intermediates in the Pr → Pfr phototransformation measured at ambient temperature and have shown that the TRCD spectrum of the lumi-R intermediate was significantly different from the steady-state CD spectrum of this intermediate trapped at low temperature. We propose that this difference is due to different protein–chromophore interactions. More specifically, we propose that lumi-R trapped in the low-temperature CD experiment[21] is a strained species, while the same intermediate undergoes partial conformational relaxation in less than about 1 μs at 10 °C. An argument based on equilibrium thermodynamics is also given to explain the fact that the partially relaxed lumi-R intermediate is not observed by low-temperature CD experiments. The above results support the proposal that changes in the structure of the chromophore pocket occur in the early stage of the phytochrome phototransformation (i.e., during the formation of lumi-R), which is consistent with earlier findings of Song et al.[35,36] The protein conformational relaxation during this time does not seem to create significant effects on the absorption spectrum, suggesting that motions of aromatic residues in the vicinity of the chromophore are more significant than motions of charged residues. The later intermediates in the phototransformation exhibit similar CD spectra, i.e., very small CD in the chromophore absorption band. We therefore suggest that there are only weak interactions between the chromophore and the nearby aromatic residues (which is most likely to give rise to the chromophore CD) in the later steps of the phototransformation.

We show in this study that TRCD spectroscopy at physiologically relevant temperatures can reveal a more accurate picture of protein structure and dynamics than conventional, steady-state CD studies in which the photointermediates are trapped at low temperatures. In this case, the first intermediate trapped at low temperatures was an unrelaxed isomerized species which could not undergo conformational relaxation due to the high activation barrier associated with the structural rearrangement of the protein. On the basis of thermodynamic arguments and the recent experimental evidence from photolysis studies on rhodopsin,[53] the partially relaxed intermediate observed by time-resolved spectroscopy at physiological temperatures cannot necessarily be trapped as a stable intermediate at low temperatures. The TRCD study presented here has also demonstrated the sensitivity of the TRCD technique to the fast changes of molecular structure in the visible part of the spectrum. This technique should be very useful as a complementary approach to time-resolved absorption studies since structural differences revealed from the TRCD spectra may not be detectable in the absorption spectra. Work is in progress to measure the absorption and CD of the initial photoprocesses of the phytochrome phototransformation using picosecond time-resolved absorption and CD.

It would be interesting to see if one could detect a fast relaxation of the chromophore pocket from the strained to the relaxed lumi-R species. It would also be very interesting to apply the TRCD technique to the phytochrome Soret and UV bands where one could follow the protein changes occurring during the phototransformation more directly. Work is currently underway to extend the TRCD technique further into the UV so that such studies will be possible.

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