spectra are nearly identical with conventional RR spectra, suggesting that interaction with the Ag surface does not significantly perturb the BVDE electronic structure. The spectrum is stable to photoisomerization and photodegradation during the laser irradiation despite resonance of the laser excitation line (413.1 nm) with the intense near-UV absorption band of the compound. The spectra of the deuterated and protonated lactam compounds were also sufficiently resolved to allow tentative band assignments.

The advantages of SERRS' sensitivity and the stabilization of the adsorbed compounds have permitted the first observation of the Raman spectra of the *E,Z,Z* and *Z,Z,E* isomers of BVDE. Differences between these *E* isomers and the corresponding *Z,Z,Z* isomer reflect the change in geometry of the molecule. Bands observed at 1245 and 1255 cm^{-1} in the *Z,Z,Z* isomer decrease in intensity upon isomerization to both of the *E* isomers. Also, the increase in intensity of the 1594-cm^{-1} band only in the *Z,Z,E* isomer helps to differentiate it from the *Z,Z,Z* isomer. These results confirm previous reports of the sensitivity of the 1245-cm^{-1} band to geometry, and specifically to configuration of the molecules. As the isomers are all reported to possess helical conformations, these bands appear to be markers for the *Z,Z,Z* configuration and specifically for the dihedral angles of rings A and D. These results will provide insight into the changes in molecular geometry associated with the phytochrome phototransformation, the study of which is described in the following article.

Acknowledgment. We gratefully thank the National Institutes of Health for support of this work, in the form of grants GM36956 (P.S.S.) and GM35108 (T.M.C.), and Richard Shoemaker for the recording and interpretation of the NMR spectra.

Surface-Enhanced Resonance Raman Scattering Spectroscopy Applied to Phytochrome and Its Model Compounds. 2. Phytochrome and Phycocyanin Chromophores†

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Abstract: Surface-enhanced resonance Raman scattering (SERRS) spectra of phytochrome at 77 K are reported. The spectra reveal significant differences between Pr and Pfr forms of phytochrome. SERRS spectra of C-phycocyanin *Z,Z,Z*- and *Z,Z,E*-chromopeptide isomers at 77 K are also reported. The phycocyanin chromopeptide studies are used to provide a basis for interpreting the phytochrome SERRS spectra. The spectra indicate that photoisomerization of chromophores from C-phycocyanin chromopeptides (from a *Z,Z,Z* to a *Z,Z,E* configuration) is detectable with SERRS. Comparison of SERRS spectra between the Pr and Pfr forms of 124-kDa phytochrome adsorbed on silver colloids demonstrates that the chromophore undergoes a $Z \rightarrow E$ isomerization during the Pr → Pfr phototransformation. However, the overall chromophore conformations are likely to be conserved for the native Pr and Pfr phytochrome species.

Phytochrome is a light receptor protein found throughout the plant world. As a plant photoreceptor, it controls and modifies a number of photomorphogenic responses, including the expression of light-responsive genes in plants.[1] The biologically inactive form (Pr) is synthesized in the dark and upon irradiation with red light is converted into an active, far-red light absorbing form (Pfr). This photoreaction is reversible.

The current model for the Pr to Pfr phototransformation of phytochrome postulates a photoisomerization around the C-15 methine bridge of the tetrapyrrolic chromophore, changing from a *Z,Z,Z* configuration in Pr to a *Z,Z,E* configuration in Pfr, as suggested from the NMR data of chromopeptides.[2] The present study provides direct evidence for *Z,Z,Z* → *Z,Z,E* isomerization in the native phytochrome protein. The Pr chromophore, which resides in a hydrophobic pocket in the protein, becomes more exposed in the Pfr form.[3] Recent work has also shown that oxidation of the chromophore with tetranitromethane takes place preferentially at the C-15 methine bridge, indicating that the protein moiety around the pyrrole D ring is sufficiently flexible to allow for some movement of this ring.[4]

C-phycocyanin, another red-light-harvesting protein, is found in blue-green algae and cyanobacteria and functions as an "antenna protein", funneling light energy into the reaction center chlorophylls. Because of the structural (Figure 1) and spectral similarities (Figure 2A,B) of C-phycocyanin to phytochrome, it has frequently been used as a model chromophore for phytochrome studies.[5]

A wealth of data has been obtained from these light-sensing chromoproteins via the use of various spectroscopic methods. From studies of model compounds with similar spectral characteristics (e.g., biliverdin, bilatrienes-*abc*), the chromophores of both the phytochrome and C-phycocyanin proteins have been identified

† Part 1, hereafter referred to as paper 1: Holt, R. E.; Farrens, D. L.; Song, P.-S.; Cotton, T. M. *J. Am. Chem. Soc.*, preceding article in this issue. The present paper was presented in part at the 16th Annual Meeting of the American Society for Photobiology.[9b]

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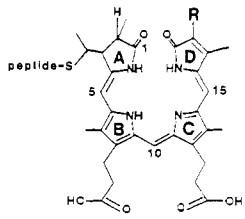
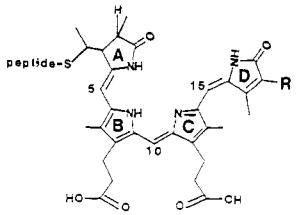


A)Z,Z,Z



B)Z,Z,E

Figure 1. Structure of C-phycocyanin and phytochrome chromophores. (A) Z,Z,Z configuration. (B) Z,Z,E configuration. R = $CH{=}CH_2$ for phytochrome, R = $CH_2{-}CH_3$ for C-phycocyanin.

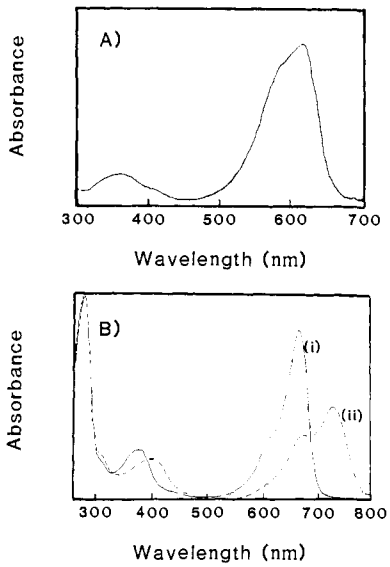


Figure 2. (A) Absorption spectrum of C-phycocyanin in 20 mM KPB, pH 7.8, 298 K. (B) Absorption spectra of phytochrome in 20 mM KPB, pH 7.8, 298 K. (i) Pr and (ii) Pfr forms. The specific absorbance ratio [absorbance at 666 nm to (protein) absorbance at 280 nm] of this sample is 0.88.

as tetrapyrrolic, bilin-type structures[6] that are attached at ring A via a C-3′ thioether bond to a cysteine residue in the protein.[7] In fact, the only structural difference between the two chromophores is the substitution of a vinyl group in phytochrome for an ethyl group in phycocyanin at C-18 (see Figure 1).

Resonance Raman (RR) spectroscopy, with its inherent ability to discriminate chromophore vibrational spectra from background spectra arising from various protein vibrations, is an ideal method for studying the structure of these chromophores. Recently, Fodor et al. reported the RR spectrum of the Pr form of phytochrome.[8] In addition, we are able to report the first vibrational spectra of both the Pr and Pfr forms of phytochrome by utilizing surface-enhanced resonance Raman scattering (SERRS) spectroscopy. Since the SERRS spectra were first presented,[9] we have undertaken a series of spectroscopic studies using both free chromophore models (paper 1) and model chromopeptides to aid in the interpretation of the phytochrome spectra.

Experimental Section

Phycocyanin Chromopeptides. C-phycocyanin from *Spirulina platensis* was obtained from Sigma Chemical Co., St. Louis, MO. Chromopeptides from C-phycocyanin were prepared by the method of Thümmler et al.[2a] Briefly, this method consists of digestion of the phycocyanin with pepsin (Sigma) at a 10:1 ratio (w/w) and then purification of the resulting chromopeptides by gel filtration on Bio-Gel P-10 and Merck silica gel columns, using 5% and then 10% HCOOH as the eluent. SERRS was performed on the mixture of the chromopeptide fractions (containing the different subunits of C-phycocyanin), without further purification steps, as the absorption spectrum and photochemical properties have been shown to be affected only slightly, if at all, by the amino acid composition of the chromopeptides.[2a] *Z-E* isomerization of the purified *Z,Z,Z*-chromopeptides was accomplished by adding β-mercaptoethanol (Aldrich) at a concentration of 40% (w/w) to the chromopeptide mixture and then irradiating with white light for 5 min under an argon atmosphere at 4 °C. Separation of the resulting *Z,Z,E*-chromopeptide isomers from the unreacted *Z,Z,Z*-chromopeptides was performed by passing the mixture of isomers through the same columns as mentioned above (using dilute HCl at pH 2.5 as the eluent). The *Z,Z,Z* isomers are preferentially retained by the Bio-Gel P-10 column. The silica gel column (which contained the bound *Z,Z,E*-chromopeptides eluted from the Bio-Gel P-10 column) was washed extensively with the dilute HCl, as it was found that residual β-mercaptoethanol interferes with SERRS spectra. All work was performed under very dim green light to avoid back-isomerization of the *E*-chromopeptides.

Phytochrome and Chymotryptic Digestion. Native 124-kDa oat phytochrome[10] was isolated from etiolated Garry oat seedlings (*Avena sativa*), according to the method of Chai et al.[11] This method yielded phytochrome with SAR values[12] ranging from 0.75 to 1.00; these samples were used directly in the SERRS studies. Chymotrypsin for the phytochrome digestion was purchased from Sigma. Proteolytic digestion of the Pr form of phytochrome was performed as described by Kwon[13] using a chymotrypsin/phytochrome ratio of 1:100 (w/w) and allowing the mixture to react for 40 min at room temperature.

Absorption Spectroscopy. Absorption spectra were recorded with a Hewlett-Packard diode array spectrophotometer (HP 8451 A) equipped with floppy disk storage capabilities. Spectra were recorded at 298 K unless otherwise indicated. Photoconversion of the samples was accomplished by irradiating at the appropriate wavelengths (either 660 nm to produce Pfr, or 730 nm to produce Pr), using an optical fiber system as described by Eilfeld and Rüdiger.[14]

SERRS Spectroscopy on Silver Electrodes. All SERRS spectra were recorded on a SPEX Triplemate monochromator/spectrograph using backscattering optics (for a more detailed description of the experimental design and instrumentation, see paper 1). All protein samples were dialyzed (to remove any residual β-mercaptoethanol) and dissolved in 20 mM potassium phosphate buffer (KPB), with 1 mM EDTA at pH 7.8 unless noted otherwise. Samples with OD_{666} ranging from 0.05 to 0.30 were adsorbed onto the electrochemically roughened electrode surface under dim green light at 4 °C or under constant irradiation with the appropriate actinic light at room temperature (either 660 nm for conversion to Pfr, or 730 nm for conversion to Pr). Spectra were recorded at 77 K (in liquid nitrogen) by use of a laser excitation wavelength of 413.1 or 676.4 nm. Twenty-five spectra were accumulated and averaged to eliminate noise resulting from nitrogen bubbling. All experiments were performed under dim green light or in total darkness to prevent back-isomerization of the *Z,Z,E*-chromopeptides.

The 413.1-nm line of a Kr^+ laser was chosen as the excitation wavelength because it is in resonance with the near-UV absorption band of both phytochrome and phycocyanin (see Figure 2). Spectra were measured at low temperatures to obtain better resolution and to eliminate the possibility of the photodegradation that occurs with excitation at room temperature. At 77 K, no significant photodegradation was observed over the period of the measurement (usually <1 min). Furthermore, the liquid

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nitrogen prevents photochemical conversion and photocycling, as the photoconversion of either Pr or Pfr is known to be blocked at temperatures below −100 °C.[14]

Preparation of Silver Colloids (Sols) and SERRS Spectroscopy. Silver colloids (sols) were prepared by the method of Lee and Meisel.[15] Reduction of silver nitrate using sodium citrate is preferred over borohydride reduction as explained by Kelly et al.[16] Sols produced in this laboratory exhibited an absorbance maximum at 406 nm and were stable for a period of weeks. As with the silver electrodes, any β-mercaptoethanol present in the phytochrome solutions was found to completely abolish the surface-enhancement effect. In order to "activate" the sol, 75 μL of 1% ascorbic acid was added to 2.9 mL of sol prior to protein addition. The pH of the sol was then between pH 5.5 and 6.0. The slightly acidified sols were chilled to 0 °C on ice before addition of the phytochrome samples.

The phytochrome solutions (OD_{666} = 1.4, SAR = 0.92) were then converted to either the Pr or Pfr forms by irradiation with the appropriate actinic light. Aliquots (3 μL) of the phytochrome solutions were then added to the chilled, slightly acidified sol solution. Immediately after addition of the phytochrome, 200 μL of 200 mM KPB was added to the sol/phytochrome solution to neutralize the solution to pH 7.0–7.5. The final concentration of the phytochrome in the sol was approximately 10 nM. Prior to freezing, the sol/phytochrome solutions were mixed with approximately 1 mL of glycerol in order to stabilize the sol as it freezes.

SERRS measurements of these sol solutions were performed in an NMR tube that was half-filled with the sol/glycerol/phytochrome solution and placed in a −70 °C freezer. This enabled the sol to form a uniform glass before immersion in liquid nitrogen (77 K). All manipulations were performed in the dark or under dim green light. The conditions used for the SERRS/sol spectroscopy were the same as in the SERRS with electrodes.

Results

Phycocyanin Chromopeptides. As noted in the introduction, the phycocyanin chromophore has often been studied as a model for phytochrome, as its structure is known to be very similar to that of phytochrome.[17] Thus, the phycocyanin chromophore is a good control for subsequent phytochrome studies.

The SERRS spectra of the C-phycocyanin *Z*- and *E*-chromopeptides (Figure 3) were examined to determine if differences between *Z* and *E* isomers can be observed with SERRS and whether adsorption onto a silver metal surface might perturb the configurational structure of the chromophores, causing a back-isomerization of the C_{15}–C_{16} *E* double bond to a C_{15}–C_{16} *Z* bond. Unfortunately, it was not possible to obtain RR spectra of these two chromopeptides for comparison with the SERRS spectra because of strong fluorescence interference. However, major differences are observed in the SERRS spectra of the *Z,Z,Z* and *Z,Z,E* isomers, including dramatic changes in the relative intensities of several bands in the spectra. Similar differences were observed for *Z* and *E* isomers of BVDE (paper 1). Of particular interest is the intensity loss of the 1259-cm^{-1} band in the *E* isomers. This band is enhanced in the SERRS of *Z,Z,Z* forms of tetrapyrroles and decreases in intensity in the *Z,Z,E* isomers (see BVDE results in paper 1). Hence, the SERRS results are a confirmation of the stability of the *Z* and *E* configurations on the Ag surface.

In conventional RR spectroscopy, a band at 1245 cm^{-1} decreases in intensity when native C-phycocyanin is denatured and the chromophore assumes a cyclohelical conformation.[18] Consequently, the 1245-cm^{-1} band has been identified as a potential marker for cyclohelical conformations in tetrapyrrolic chromophores. However, in the SERRS spectra of the C-phycocyanin chromopeptides the 1259-cm^{-1} band enhancement (Figure 3) masks the 1245-cm^{-1} band, making it difficult to draw definitive conclusions regarding the chromophore conformation by use of

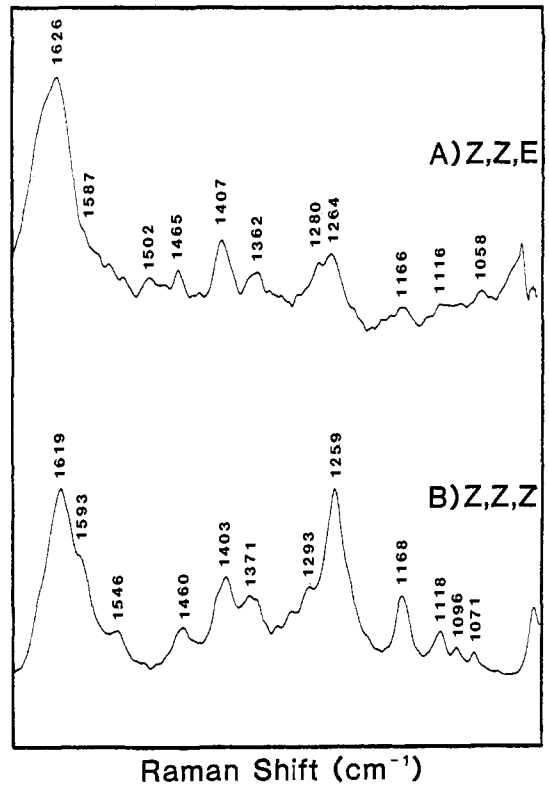

Figure 3. (A) SERRS/electrode spectrum (77 K) of C-phycocyanin *Z,Z,E*-chromopeptide. Laser power, 60 mW; band pass, 3 cm^{-1}, excitation wavelength, 413.1 nm. Adsorbate concentration, ≈10^{-5} M. (B) SERRS/electrode spectrum (77 K) of C-phycocyanin *Z,Z,Z*-chromopeptide. Laser power, 50 mW; band pass, 3 cm^{-1}; excitation wavelength, 413.1 nm. Adsorbate concentration, ≈10^{-5} M.

variations in 1245-cm^{-1} band intensity alone. In the *E* isomers of BVDE, a 1258-cm^{-1} band is also decreased in intensity (paper 1), and therefore, the 1259-cm^{-1} band can be used as a marker to differentiate between *Z,Z,Z* and *Z,Z,E* chromophores.

In an attempt to assign frequencies to specific vibrational modes, SERRS spectra of C-phycocyanin were recorded in D_2O and in H_2O (not shown). A shift of the 1259-cm^{-1} band in D_2O was apparent, and we tentatively attribute this shift to a loss in intensity of the 1245-cm^{-1} band, which leads to an apparent shift in the maximum of the 1259-cm^{-1} band. Similar shifts were observed in BVDE (paper 1).

Phytochrome. Phytochrome on Silver Electrode Surfaces. To determine whether the purported $Z \rightarrow E$ isomerization in phytochrome could be observed on the electrode surface, the following experiments were performed. First, the SERRS spectra of both the Pr and Pfr forms of phytochrome were recorded, following room-temperature adsorption of the protein onto the electrode. In a second experiment, the two forms were adsorbed at 0 °C in an attempt to stabilize the protein structure. Finally, in a third experiment, the Pr form was deliberately digested with chymotrypsin to expose the chromophore. After the SERRS spectrum of this Pr species was taken, the sample was irradiated with 660-nm light to produce a bleached Pfr form, and the two spectra compared. The results obtained for these three cases are described below.

Figure 4 illustrates the SERRS spectra of the Pr and Pfr forms of phytochrome at 77 K. The protein was adsorbed on the Ag electrode at room temperature. On initial inspection, major differences between the Pr and Pfr forms of phytochrome are not apparent. At most, only slight differences in intensities and small frequency shifts are seen.

Of more consequence are the significant differences that were observed between this spectrum (Figure 4) and spectra obtained under changed experimental conditions. Intense, highly resolved spectra, as shown in Figure 4, were obtained by adsorbing phytochrome samples kept at 4 °C onto the electrode at room tem-

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(18) Denaturation and renaturation experiments with phycocyanin have indicated that the phycocyanin chromophore (which is of a *Z,Z,Z* configuration) exhibits a predominantly helical conformation in denatured proteins and an extended conformation in native proteins.

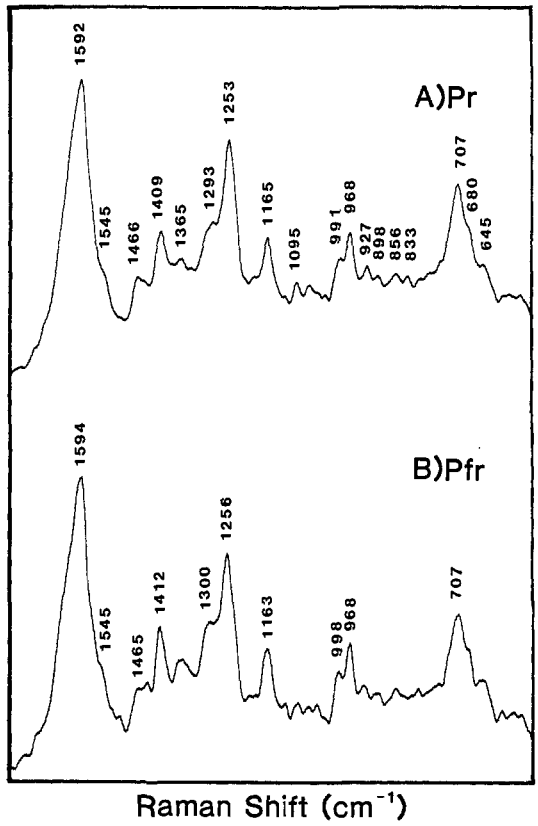

Figure 4. (A) SERRS/electrode spectrum (77 K) of phytochrome, Pr form. Laser power, 100 mW; band pass, 3 cm^{-1}; excitation wavelength, 413.1 nm. Adsorbate concentration, $\approx 10^{-6}$ M. (B) SERRS/electrode spectrum (77 K) of phytochrome, Pfr form [same conditions as used in (A)]. Samples were adsorbed from 20 mM KPB solutions, pH 7.8, for 15 min at room temperature while irradiating with actinic light.

perature (23 °C) for 15 min during constant irradiation with either 730- or 660-nm light. The sample is thus converted to the Pr or Pfr form prior to immersion in liquid nitrogen. In contrast, much poorer and less reproducible spectra were obtained when the adsorption was performed at 0 °C (on ice) under dim green light (spectra not shown). This may suggest that the chromophore is more distant from the electrode surface when the protein is adsorbed at 0 °C. A slightly lower relative intensity of the 1259-cm^{-1} band under these conditions might also be of significance, implying that the chromophore has a different conformation than that possessed by the samples adsorbed at room temperature. The weaker spectra seen under these conditions could also be due to other factors, such as aggregation of the phytochrome at lower temperatures.

The objective of the chymotrypsin digestion of phytochrome (Figures 5 and 6) was to determine whether the SERRS spectrum of a highly degraded 39-kDa chromopeptide resembled the SERRS spectrum of phytochrome adsorbed on the electrode at room temperature. The rationale of this procedure was as follows: it is impossible to test directly for phytochrome denaturation as determined by the loss of its native absorption spectrum on the electrode surface. Therefore, "native" SERRS/electrode phytochrome spectra (Figure 4) were compared with purposely denatured, digested phytochrome SERRS/electrode spectra (Figure 6) to look for similarities or differences. Figure 5 shows the absorption spectra of the Pr form of phytochrome before (5A) and after (5B) a 40-min chymotryptic digestion, followed by irradiation of the digest with 660-nm light to produce a bleached, cyclic Pfr form (5C). This latter species exhibits the characteristic absorption spectrum of a cyclic tetrapyrrolic chromophore and closely resembles the cyclic/helical Pbl_{640} previously reported.[19]

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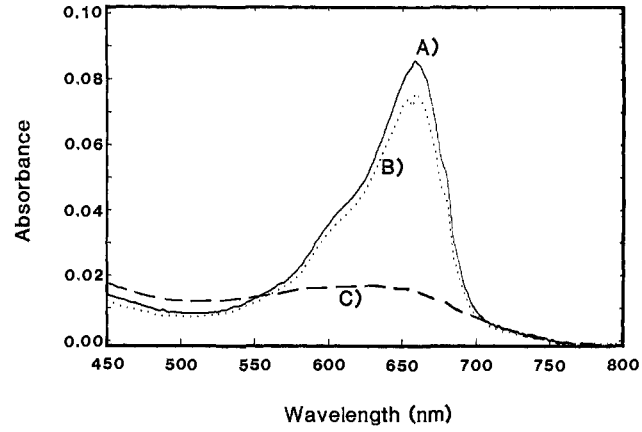

Figure 5. Absorption spectra of chymotryptically digested phytochrome in 20 mM KPB, pH 7.8, 298 K. (A) (—) before digestion with chymotrypsin. (B) (···) after 40-min digestion with chymotrypsin, prior to irradiation with 660-nm light. (C) (- - -) after irradiation of (B) with 660-nm light to form bleached "Pfr" form (Pbl_{640}). OD_{666} = 0.22 for the initial Pr form, 1-mL total volume (see Experimental Section).

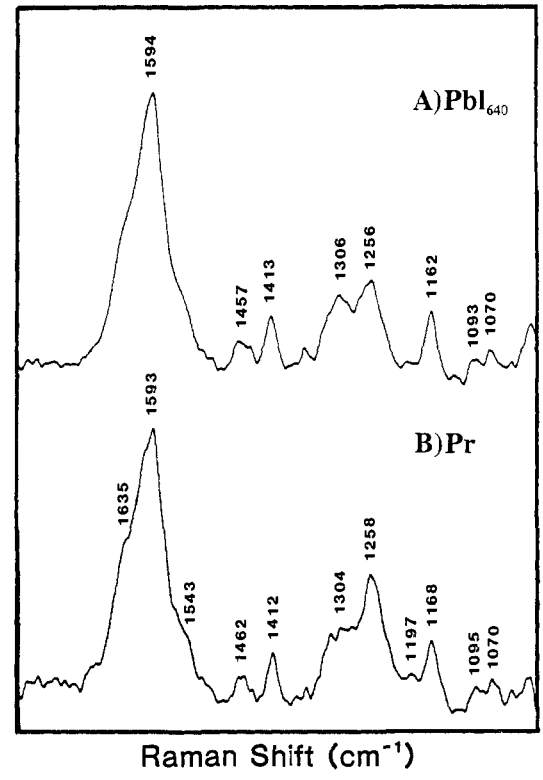

Figure 6. (A) SERRS/electrode spectrum (77 K) from Figure 5, Pbl_{640} form from chymotryptic digestion of the Pr form, followed by 5-min irradiation with 660-nm light (for more details see Experimental Section). (B) SERRS/electrode spectrum (77 K) of chymotryptic digested Pr form. Laser power, 100 mW; band pass, 3 cm^{-1}, excitation wavelength, 413.1 nm. Adsorbate concentration, $\approx 2 \times 10^{-6}$ M in both spectra.

Figure 6 shows the SERRS spectrum of the digested Pr and bleached Pfr (Pbl_{640}) forms.

The Pr absorption spectra are very similar before and after chymotryptic digestion (Figure 5), indicating that after proteolytic degradation the chromophore in the Pr peptide species retains its native noncyclic, semiextended conformation.[13] Furthermore, the SERRS spectrum of this degraded Pr phytochrome is also similar to the original nondegraded Pr SERRS spectrum (compare Figures 4A and 6B). These data indicate that chymotryptic digestion does not significantly disrupt the native protein–chromophore interactions in the Pr form of phytochrome.

The absorption spectrum of native Pfr (Figure 2) indicates that the chromophore has a semiextended conformation.[20] When the

chymotryptic Pr chromopeptide digest is irradiated with 660-nm light, a species is produced that exhibits the characteristic (cyclic) Pbl_{640} absorption spectrum[13,19] (Figure 5C). Notice that this denatured, cyclized species exhibits a SERRS spectrum (Figure 6A) different from that obtained from the undigested Pfr form (Figure 4B). The spectral significance of this digested, bleached Pfr phytochrome is discussed below.

All of the SERRS spectra obtained from phytochrome adsorbed at room temperature were highly reproducible. The possibility that the SERRS/electrode phytochrome spectra are artifactual and not representative of the unperturbed, native phytochrome chromophore must be considered. The origins of possible artifacts and their consequences for the SERRS/electrode phytochrome spectra (Figure 4) are analyzed below.

Instability of *E* Isomers on the Electrode Surface. Initially, there was concern that *E* isomers might revert back to a *Z,Z,Z* form upon adsorption onto the electrode surface, due to an inherent instability of an *E* isomer on a Ag surface. However, the fact that SERRS spectra show distinct differences between (*Z*)- and (*E*)-BVDE isomers (paper 1) and that similar differences are also observed for the C-phycocyanin *Z*- and *E*-chromopeptide isomers provides evidence that the *E* isomers are stable on the Ag electrode surface.

Photoisomerization of the Isomers. The possibility that photoisomerization may occur during the measurement is of concern because, in general, *E* isomers are easily photoconverted back to their more stable *Z,Z,Z*-isomer form. However, as noted above, differences between *E* and *Z* isomers are observed in the SERRS spectra of BVDE and the phycocyanin chromopeptides. This indicates that no significant photoisomerization occurred under the conditions of the experiment. If photoisomerization had occurred, a photoequilibrium mixture would result and there would be no difference between the SERRS spectra of the two forms. As can be seen in Figure 3, this is not the case and it can be concluded that back-isomerization of the *Z,Z,E* isomer to the *Z,Z,Z* form does not occur to any significant degree during these measurements.

Denaturation or Dark Reversion. One interpretation for the similarity of the Pr and Pfr SERRS spectra in Figure 4 is that adsorption onto the electrode surface denatures the proteins, disrupts the protein–chromophore interaction, and allows the chromophores to change from their normally semiextended conformation into similar cyclohelical forms. This explanation for the similarity of Pr and Pfr phytochrome spectra is not entirely adequate for the following reasons:

(a) Digested, denatured C-phycocyanin *E*- and *Z*-chromopeptides show significant differences in their SERRS spectra. If Pr and Pfr phytochromes were denatured upon adsorption, resulting in chromophore cyclization like that of a free tetrapyrrole, the differences observed between the SERRS C-phycocyanin spectra of *Z,Z,Z*- and *Z,Z,E*-chromopeptides should be seen in the phytochrome Pr and Pfr SERRS spectra. Instead, nearly identical Pr and Pfr phytochrome spectra (Figure 4A,B) are observed. The similarity of these spectra and, more specifically, the high relative intensity of the 1259-cm^{-1} band in the Pfr form (previously observed only for *Z,Z,Z*-tetrapyrroles), indicates that the SERRS spectrum of the Pfr form of phytochrome cannot be that of a cyclized *E* chromophore. This conclusion is based upon the observation that *E* isomers from C-phycocyanin chromopeptides and from BVDE are stable and distinctly different from *Z,Z,Z* isomers on an electrode surface. Additionally, previously observed NMR and absorption spectra of the BVDE *E* isomers (paper 1) were used to identify and confirm them as such.

A denaturation-based explanation for the similar phytochrome SERRS/electrode spectra would be valid only if it were assumed that a *Z* to *E* isomerization does not occur during the phytochrome phototransformation and that both Pr and Pfr have a *Z,Z,Z* configuration. Such a conclusion appears plausible and would agree with the SERRS/electrode data presented here. However, to say *Z* to *E* isomerization does not occur in the photoconversion of phytochrome is contrary to the NMR evidence for photoisomerization of the Pr and Pfr chromopeptides.[21] An explanation that is more consistent with the current body of phytochrome data is that no denaturation-induced cyclization of the chromophore has taken place, and that instead, the Pfr form reverts thermally (dark reversion) to the Pr form during the adsorption onto the silver electrode at 23 °C. Dark reversion of phytochrome from Pfr to the more thermodynamically stable Pr form is a well-known phenomenon[22] that can be accelerated by various factors, especially the adsorption of phytochrome to antibodies. This would also account for the fact that the SERRS spectra of the samples adsorbed at 0 °C differ from those adsorbed at 23 °C.

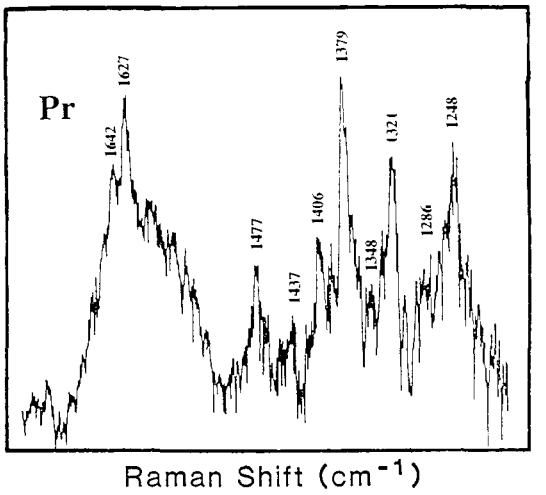

Figure 7. SERRS/sol spectrum (77 K) of phytochrome, Pr form. Laser power, 50 mW; band pass, 3 cm^{-1}; excitation wavelength, 676.4 nm. Adsorbate concentration, $\approx 10^{-8}$ M.

(b) The most likely mechanism for a hypothetical unfolding/denaturation of a protein during SERRS adsorption is the formation of Cys–S–Ag bonds on the electrode surface, as thiol groups have been shown to have a high affinity for Ag electrode surfaces.[23] This type of unfolding/denaturation would probably not drastically affect the SERRS spectrum of phytochrome, because the phytochrome chromophore shows only slight spectral denaturation/conformational change upon modification of its most accessible protein SH groups.[24]

(c) Considering these last two points together with the SERRS data from the chymotrypsin digestion, we conclude that adsorption onto the electrode surface does not cause denaturation and/or cyclization of the phytochrome chromophore, but that there is significant thermal reversion of the Pfr form to the Pr form on the electrode surface. The reproducible SERRS differences observed between the Pr and Pfr forms (Figure 4) are likely attributable to an unreverted residual fraction of the Pfr form.

Phytochrome on Colloidal Silver Sols. Since the major differences in the SERRS spectra observed between *Z* and *E* isomers of the model compounds were not observed in our phytochrome–electrode system, most likely due to dark reversion of the Pfr form accelerated by the electrode adsorption at room temperature, we employed silver colloids as the SERRS substrate.

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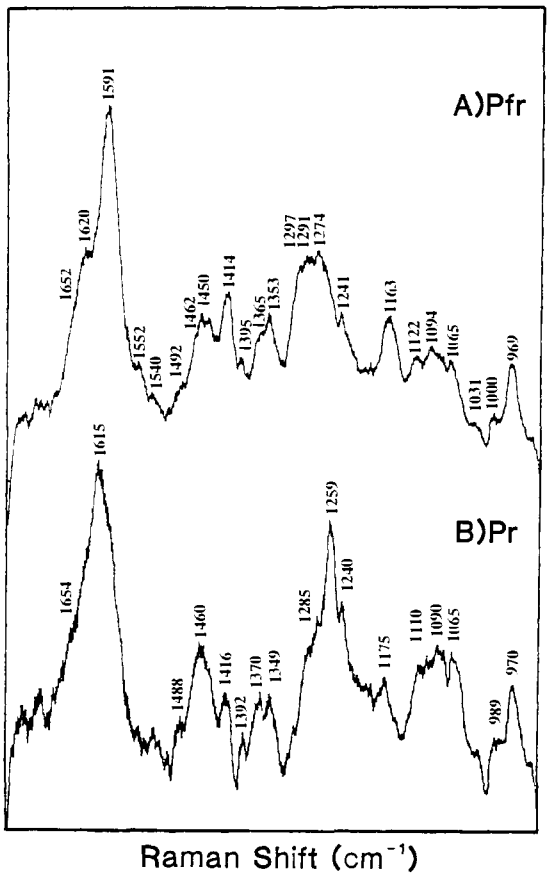

Figure 8. (A) SERRS/sol spectrum (77 K) of phytochrome, Pfr form. Laser power, 100 mW; band pass, 3 cm^{-1}; excitation wavelength, 413.1 nm. Phytochrome (3 μL) OD_{666} = 1.4 in 20 mM KPB, pH 7.8, was added to 2.9 mL of silver sol (see Experimental Section) on ice (0 °C) under dim green lights. Adsorbate concentration, $\approx 10^{-8}$ M. (B) SERRS/sol spectrum (77 K) of phytochrome, Pr form [same conditions as used in (A)].

Silver colloids were chosen because they have been demonstrated conclusively to retain biological activity/integrity of hemoglobin,[25] cytochrome *c*[26] (at 77 K), and cytochrome P-450[27] proteins adsorbed on the silver sol surface. This may in part be due to the possibility that the silver surface of the sols is covered with citrate, thus shielding the protein from direct contact with the metal surface.

We confirmed the absence of denaturation of phytochrome on the sols by comparing our Pr SERRS/sol spectra obtained using 676.4-nm excitation (Figure 7) with the reported RR spectrum of Pr phytochrome.[8] The spectra are very similar. Our Pr SERRS/sol spectrum at 676.4 nm (Figure 7) exhibits prominent peaks at 1248, 1321, 1379, 1406, 1477, 1627, and 1642 cm^{-1}, compared with the RR spectrum of Pr[8] in the same wavenumber range, which exhibited prominent peaks at 1240, 1326, 1378, 1408, 1475, 1576, 1626, and 1644 cm^{-1}. However, the SERRS/sol spectrum was of poorer quality due to the fluorescence background inherent with 676.4-nm excitation. We were unable to use the 752-nm excitation line as used by Fodor et al.,[8] due to the poor sensitivity of our diode array detector in the far-red. Attenuation of the surface-enhancement effect by the silver sols also occurs at this long wavelength.

The SERRS spectra of phytochrome on the silver sol is shown in Figure 8 for 413.1-nm excitation. A significant decrease of the 1259-cm^{-1} mode is observed in the Pfr (8A) compared with the Pr spectrum (8B), as is observed in the model chromophore *Z*/*E* isomers (vide supra; paper 1). There are other gains in relative intensity of the higher frequency modes for the Pfr form at 1414, 1450, 1591, and 1620 cm^{-1}. A prominent new peak appears at 1163 cm^{-1} in the Pfr form along with intensification of other less prominent modes. For example, an increase in intensity of a peak at 857 cm^{-1} is also observed (not shown).

The high relative intensity of the 1259-cm^{-1} band in the Pr SERRS/sol spectrum (Figure 8B) is also observed in the Pr SERRS/electrode spectrum (Figure 4A). However, the Pfr SERRS/sol spectrum (8A) exhibits a pronounced decrease in the 1259-cm^{-1} peak as compared to the Pfr SERRS/electrode spectrum (4B).

Discussion

It is important to note that the ability of RR and SERRS spectroscopy to detect differences between *Z* and *E* isomers is not entirely due to shifts in the frequencies associated with the isomerization of the double bond itself. Differences between the calculated vibrational frequencies at the C_{15}–C_{16} double bond of the *Z*,*Z*,*Z* and *Z*,*Z*,*E* forms of a phycocyanobilin model chromophore are predicted to vary.[28] The observed changes in vibrational frequencies and intensities are induced by the different conformations that the chromophores are forced to assume, due to the steric effects that are associated with the *Z*–*E* isomerization. Thus, the spectral differences can only be attributed to an overall conformational change that accompanies a *Z*–*E* isomerization, *not* simply to the difference in vibrational frequencies between a C_{15}–C_{16} *E* bond and a C_{15}–C_{16} *Z* bond.[29]

An interpretation for the differences in the SERRS spectra of *Z* and *E* forms of tetrapyrroles is as follows. Calculated results[30] and crystal structure analysis of model compounds[31] have shown that a discernible difference exists in the exocyclic dihedral angles of helical *Z* isomers as compared to *E* isomers. The *Z*,*Z*,*Z* forms have been predicted (and shown) to adopt a fully cyclic conformation in free chromophores, exhibiting little difference in their exocyclic dihedral angles. Thus, their overall conformation (and π-conjugation network) is essentially helical. On the other hand, the exocyclic dihedral angles of *E* forms are twisted approximately 20° greater than in their *Z* forms. This twist may perturb the π conjugation of the system sufficiently to explain the observed differences in the SERRS spectra of the *E*-chromopeptides and *Z*-chromopeptides of C-phycocyanin. This is seen most notably in the decrease of the 1259-cm^{-1} band. Another result of the change in the exocyclic dihedral angle may be a less effective overlap in the Franck–Condon factors, thereby producing the decrease in relative intensity observed in the 1259-cm^{-1} band. This interpretation is supported by recent work of Schneider et al.[32] in which C-phycocyanin from *Mastigocladus laminosus* was treated with *p*-(chloromercuri)benzenesulfonate (PCMS). PCMS binds to a cysteine residue located in the vicinity of the chromophore. In these experiments, the marker 1245-cm^{-1} band was shown to be sensitive to changes in chromophore conformation. The 1245-cm^{-1} band disappeared upon treatment with PCMS, and this was attributed to a change in the conformation at ring

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(29) A good example of this is the difference observed in the RR of phycocyanin before and after denaturation.[18] The only change is in the conformation; the configuration of the chromophore remains *Z*,*Z*,*Z*. Also see the recent CARS work of Schneider et al.,[32] where they observed a change in the spectrum upon changing the conformation/geometry of the B-84 chromophore by titrating the neighboring cysteine B-111 with PCMS.
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D, possibly due to the rotation of this ring out of the planarity of the π system. In SERRS, the 1245-cm^{-1} band lies underneath the 1259-cm^{-1} envelope, but undoubtedly contributes to its overall intensity. A parallel can easily be drawn between the disappearance of the 1245-cm^{-1} band in the cited work and the disappearance of the 1259-cm^{-1} band seen in the present study, wherein SERRS indicates *Z–E* isomerization of C-phycocyanin chromopeptides and BVDE, both of which also involve rotation of an exocyclic ring.

Another interesting observation is that, in the case of the BVDE *E* isomers, only the *Z,Z,E* isomer (paper 1) showed a dramatic increase in the 1594-cm^{-1} band, analogous to the increase in the band at 1591 cm^{-1} observed in the Pfr SERRS/sol phytochrome spectrum. The relative intensity of the 1594-cm^{-1} band did not increase in the *E,Z,Z* isomer of BVDE. This adds support for the proposed Pr to Pfr phototransformation model involving a *Z–E* isomerization at the C_{15}–C_{16} double bond. By using the C-phycocyanin *Z*- and *E*-chromopeptide models as a control, we have verified that no additional or artifactual photoisomerization occurs due to our experimental measurement conditions.

The proteolytically denatured, cyclized *Z,Z,E* Pfr form (Figure 6A) shows the same decrease in the 1259-cm^{-1} band as in the C-phycocyanin *E*-chromopeptides and (*Z,Z,E*)-BVDE. This decrease in band intensity at 1259 cm^{-1} can be considered a good indication of the conformational changes that accompany a *Z–E* isomerization in free tetrapyrroles. The decrease in this band might then be attributed to a change in conformation and possible disruption of the π network caused by the twisting of the C_{14}–C_{15} dihedral angle that occurs upon *Z–E* isomerization of the free tetrapyrrole. The 1259-cm^{-1} band seems to contribute to the intensity of the large band envelope observed in this region of the native Pfr SERRS spectrum (Figure 8A), suggesting that the twisting of ring D is less in the native Pfr protein than in the free chromophore. A decrease in the twisting of the D ring in Pfr, with the subsequent increase of the π-conjugation network might offer an explanation for the long-wavelength shift observed for the Pfr chromophore in the native protein.

The vibrational frequencies from the SERRS/electrode for the Pr and Pfr chromophores (Figure 4) are slightly different. Denaturation of the protein adsorbed on the electrode surface cannot be ruled out, but the reproducible differences in vibrational frequencies between the two phytochrome forms can be explained in terms of the substantial dark reversion of the Pfr species on the electrode surface, with the residual Pfr population contributing to the observed small differences in the SERRS/electrode spectra. We favor this interpretation over the adsorption-induced denaturation of phytochrome, particularly in the case of the Pfr species, because denaturation of the protein would have produced a SERRS/electrode spectrum of the Pfr form similar to that shown in Figure 6A.

Dark reversion does not occur with the sols most likely because citrate covers the silver surface, and because the phytochrome remains in an aqueous environment in the sols. In contrast, the phytochrome in the SERRS/electrode system is in direct contact with liquid nitrogen. Thus, a dehydration of phytochrome, caused by the liquid nitrogen, may also partially account for the similarity of the SERRS/electrode phytochrome spectra.[33]

The proteolytically digested, cyclized *Z,Z,E*-Pfr form (Figure 6A) shows the same decrease in the 1259-cm^{-1} mode as in denatured C-phycocyanin *E*-chromopeptides and (*Z,Z,E*)-BVDE (paper 1). This decrease in band intensity at 1259 cm^{-1} can be considered to be a good indicator of the conformational changes that accompany a *Z–E* isomerization in free tetrapyrroles. The fact that the 1259-cm^{-1} mode in the SERRS/sol spectrum of Pfr is significantly reduced in intensity (Figure 8A) is consistent with

(33) Rüdiger et al.[2b] report that glycerol-dehydrated phytochrome yields Pr and Pfr species that exhibit nearly identical UV–vis spectra. Upon rehydration, these species regain their native spectral characteristics. The reversibility of this effect would imply that denaturation of the phytochromes does not occur upon dehydration. Hence, the similar Pr and Pfr SERRS/electrode spectra may be due to a related liquid nitrogen induced dehydration phenomenon.

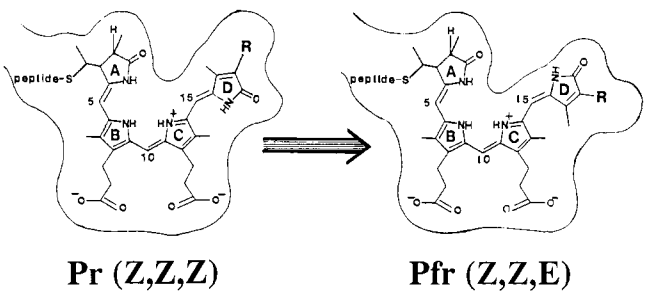

Figure 9. Cartoon model of the phototransformation of the Pr chromophore to the Pfr chromophore. The tetrapyrrolic chromophore retains an overall semiextended conformation during the transformation, with the exocyclic dihedral angle at ring **D** being conserved by a chromophore–apoprotein interaction. The protonation of ring C is from recent work by Fodor et al.[8]

the isomerization of the chromophore. The degraded 39-kDa cyclic Pbl_{640} form of degraded phytochrome gives the characteristic SERRS spectrum of an *E* isomer (Figure 6A), suggesting that it is unable to dark-revert back to the Pr *Z,Z,Z* form.

The SERRS/sol spectra of the Pr and Pfr forms of phytochrome are clearly different in several features,[34] as shown in Figure 8 and described under Results. Furthermore, since our SERRS/sol spectrum of the Pr form of phytochrome obtained with 676.4-nm excitation (Figure 7) agrees with the RR spectrum of native Pr[8], it is safe to conclude that all of the SERRS/sol spectra arise from native phytochrome. Therefore, the differences between the Pr and Pfr SERRS/sol spectra represent the inherent vibrational differences between the native protein chromophores.

Conclusions

In paper 1, it was demonstrated that differences between *Z,Z,Z* and *Z,Z,E* configurations of a phytochrome model chromophore (BVDE) can be observed by using SERRS spectroscopy. In this paper, we have shown that differences between *Z,Z,Z* and *Z,Z,E* configurations prepared from C-phycocyanin chromopeptides can also be observed, the differences being essentially the same for both model chromophores.

Although the SERRS spectra of phytochrome adsorbed on silver electrodes were obscured by the possible dark reversion of the Pfr form of the protein, the SERRS spectra obtained from silver sols yielded evidence consistent with the photoisomerization of the phytochrome chromophore.

Since detailed normal mode assignments have not been determined for the native phytochrome chromophores, it is not certain if the conformations of the Pr and Pfr chromophores are largely conserved and similar (i.e., semiextended) as a result of apoprotein–chromophore interactions. Figure 9 shows a tentative mechanism for the Pr → Pfr, *Z* → *E* photoisomerization with a conservation of the chromophore conformation in the native phytochrome. Previous studies based on oscillator strength ratios (visible to near-UV; see Figure 2) have indicated that the chromophores in both native Pr and Pfr forms possess similar semiextended conformations.[19,20] This model is consistent with a protein-controlled chromophore conformation that is similar for the native Pr and Pfr phytochromes.

Finally, it can be postulated that the conservation of the chromophore conformations in the Pr → Pfr transformation after

(34) We have dismissed the possibility that surface selection rules account for the differences observed between the SERRS spectra of the Pr and Pfr forms of phytochrome. The spectral differences observed between the Pr and Pfr forms of phytochrome are due to differences between their geometric and electronic structures. The surface selection rule effect is not as chromophore specific as the RR effect.[27] Surface selection rules may be responsible for differences between SERRS and solution RR spectra from an individual chromophore, but not for relative intensity and frequency differences between SERRS spectra from two photoisomeric chromophores embedded in a protein matrix (Rospendowski et al., to be published). Finally, while this manuscript was being reviewed an abstract appeared (Fodor, S. P. A.; Lagarias, J. C.; Mathies, R. A. *Photochem. Photobiol.* **1989**, *49*, 26S) confirming that differences exist between the conventional resonance Raman spectra of the Pr and Pfr forms of phytochrome.

the primary photoisomerization step is responsible for the finely tuned light-sensing mechanism of phytochrome. The molar absorptivities of native Pr and Pfr phytochromes are very similar (Figure 2), though the molar absorptivities of their free chromophores differ markedly. The proposed model provides for a photoreceptor that is equally sensitive to both red and far-red light and can thus respond equally well to fluctuations in the light environment of plants.

More detailed analysis of the SERRS spectra is required, along with use of different wavelength excitations. Work of this type is now in progress in this laboratory.

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Conformational Analysis. 13. Molecular Structure and Conformation of 2-Bromopropenal As Determined by Gas-Phase Electron Diffraction

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Abstract: 2-Bromopropenal has been studied by gas-phase electron diffraction at 353 K. Two conformers were identified, a more stable planar anti form and a less stable planar (or near-planar) syn form. The mole fraction of the anti form, with uncertainty estimated at 2σ, was found to be 0.64 (11). Assuming the two conformers to have equal entropy, this corresponds to an energy difference of $\Delta E° = E_s° - E_a° = 1.7 \pm 1.4$ kJ·mol^{-1}. Values of bond distances (r_a) and valence angles ($\angle_\alpha$) with estimated 2σ uncertainties are the following: r(C—H) = 1.103 (28) Å, r(C=O) = 1.219 (8) Å, r(C=C) = 1.350 (13) Å, r(C—C) = 1.498 (13) Å, r(C—Br) = 1.883 (8) Å, ∠C—C=C = 121.7 (11)°, ∠C—C=O = 124.0 (17)°, ∠C—C—Br = 116.1 (19)°, ∠C=C—H = 122 (9)°, ∠C—C—H = 115 (10)°, σ_{anti} (root mean square torsional amplitude for the anti conformer) = 25 (10)°, σ_{syn} = 27 (14)°.

Most molecules with a conjugated diene system have been found to have the double bonds anti to each other. For several such compounds, an additional form has also been observed where the double bonds are either syn or gauche to each other. Propenal[1,2] (CH_2=CH—CH=O) and glyoxal[2] (O=CH—CH=O) are mixtures of anti and syn conformers, while oxalyl chloride[3] and oxalyl bromide[4] (O=CX—CX=O, X = Cl, Br) both have a nonplanar gauche form in addition to the anti conformer. In propenoyl chloride[5] (CH_2=CH—CCl=O) and 2-chloropropenoyl chloride[6] (CH_2=CCl—CCl=O) the low-energy conformer is again the planar anti form, while the second conformer is a planar, or very nearly planar, syn form. The energy difference between the conformers is found to be reduced when an aldehyde proton is replaced by a halogen atom.

In order to further determine the conformational effects of different substituents in molecules like these, we were also interested in studying propenals substituted with a halogen atom in the 2-position. We therefore initiated an electron diffraction investigation of 2-bromopropenal (Figure 1). The results for 2-chloropropenal have since then also been published,[7] and a mixture of conformers was observed, anti with a planar or nearly planar molecule and gauche with a torsion angle of $\phi = 136 \pm 8°$ ($\phi = 0°$ for a planar anti form).

Experimental and Data Reduction

2-Bromopropenal was prepared as described by Berlande.[8] The sample was purified by several vacuum destillations and stored at −78 °C in the dark. Electron diffraction photographs were recorded at 353 K with the Oslo Balzers instrument[9,10] on Kodak electron image plates. The voltage/distance calibration was made with benzene as reference. The nozzle-to-plate distances were 500.12 and 250.12 mm for the long and the short camera experiments and the electron wavelength was 0.05864 Å. Three plates from the long and five plates from the short camera distances were selected for analysis. Optical densities were measured using a Joyce Loeble double-beam microdensitometer and the data were reduced in a way reported elsewhere.[3,11-13] The data covered the range $2.00 \le s \le 14.75$ Å^{-1} and $4.00 \le s \le 25.00$ Å^{-1} ($s = 4\pi\lambda^{-1} \sin\theta$, where λ is the electron wavelength and 2θ is the scattering angle), and the data interval was $\Delta s = 0.25$ Å^{-1}. The average experimental intensity curves in the form of $sI_m(s)$ are shown in Figure 2; data for the individual curves and backgrounds[13] are available as supplementary material. The atomic scattering and phase factors used were obtained from the tables of Schäfer et al.[14]

Structure Analysis

An experimental radial distribution (RD) curve (Figure 3) was calculated in the usual way by Fourier transformation of the function $I'(s) = sI_m(s)Z_cZ_{Br}A_c^{-1}A_{Br}^{-1} \exp(-Bs^2)$ with $B = 0.0020$ Å^2. The A's are electron scattering amplitudes multiplied by s^2. Data for the unobserved region $0 \le s \le 1.75$ Å^{-1} were at first omitted and in later calculations taken from models close to the final one.

The appearance of the radial distribution curves (the final ones are shown in Figure 3) led to trial values for the important bond distances and valence angles. These values agreed quite well with the results reported for related molecules. The experimental RD

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