Conclusions

Values of $D°(Mg^+–OH)$ = 75 ± 4 kcal/mol and $D°(Mg^+–O)$ = 53 ± 3 kcal/mol were obtained from photodissociation thresholds. $D°(Mg^+–OH)$ is in excellent agreement with Murad's experimental results[4,5] indicating that the photodissociation thresholds for MgL^+ species can yield absolute bond energy information. The IP(MgOH) = 7.3 ± 0.1 eV was also determined by photodissociation experiments, while IP(MgO) = 7.9 ± 0.1 eV was determined by charge-transfer reactions. With the use of these experimental results, a variety of other thermochemical values for MgOH and MgO were obtained and summarized in Table VI. Also, our results are in excellent agreement with the ab initio calculations[9] on MgO and may imply that the experimental results obtained from both flame measurements[23,24] and Knudsen cell mass spectrometry[26] are high, possibly due to sample impurities.

In view of these results, the use of photodissociation experiments continues to play an important role for the determination of organometallic bond energies.

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Surface-Enhanced Resonance Raman Scattering Spectroscopy Applied to Phytochrome and Its Model Compounds. 1. Biliverdin Photoisomers

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Abstract: The application of surface-enhanced resonance Raman scattering (SERRS) spectroscopy to the analysis of the configuration of biliverdin dimethyl ester (BVDE) is reported. SERRS spectra obtained by adsorption of the compounds onto an electrochemically roughened silver electrode and recorded at 77 K were intense and free of significant photodegradation. The similarity of the SERRS and resonance Raman (RR) spectra obtained under identical conditions suggests that no perturbation of the electronic structure of the BVDE occurs upon interaction with the silver surface, and that the distribution of conformers comprising the BVDE solution is not changed. SERRS spectra of the deuterated and monoprotonated *Z,Z,Z* isomer are also presented. To investigate the influence of configuration upon the Raman spectrum we have synthesized and purified the *E,Z,Z* and *Z,Z,E* isomers of BVDE. Excellent SERRS spectra were obtained from the solutions of the compounds eluted directly from the TLC plates. Comparison of these isomers to the more stable *Z,Z,Z* isomer reveals differences in frequencies and relative intensities associated with the change in molecular geometry upon isomerization. Most significant is the change in intensity of bands observed at 1255 and at 1245 cm^{-1}, a band that has been previously correlated to changes in conformation of the closely related phycocyanin chromophore.

Biliverdin, a bile pigment, has attracted attention recently as a model compound for the chromophores associated with the light-harvesting antenna pigments known as the phycocyanins, which are found in red algae, cyanobacteria, and cryptophytes, and the photoreceptor pigments associated with photomorphogenesis (phytochrome in higher plants) and chromatic adaptation ("adaptachromes" in cyanobacteria and red algae).[1] The structures of the phycocyanin chromophores are relatively well characterized for only a few phycobiliproteins. The structure of the red-absorbing form of phytochrome, Pr, has been established with confidence[2] and that of the far-red-absorbing form, Pfr, with less confidence.[3] In both of these types of biliproteins, the configurations about the C=C double bonds and the conformation of the molecule produced by rotation about the single C—C bonds appear to be extremely important in producing the spectroscopic properties required by the organism for the biliproteins' specialized function. Consequently, biliverdin has become the focus for a number of spectroscopic investigations[4] and theoretical calculations[5] aimed at providing insight into the dependence of the spectroscopic properties of these biliverdin-type chromophores upon their molecular geometry.

Resonance Raman (RR) spectroscopy has enjoyed success as a method of analysis of chromophore-containing molecules of biological interest.[6] The combination of excellent sensitivity and selectivity for only the chromophoric group allows vibrational spectra to be observed in solutions of relatively low concentrations

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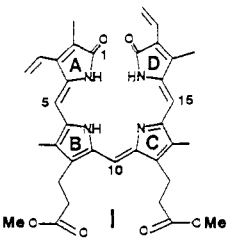
without interference from the associated nonchromophoric material (e.g., surrounding protein matrix, buffer components). Additionally, the judicious choice of the laser excitation wavelength provides an additional degree of selectivity when a number of different chromophores are present in the system.

Recently, RR spectroscopy has been applied to the study of the phycocyanins. Interference from the fluorescence that is characteristic of these compounds has been minimized by laser excitation (363.8 nm) and detection in a region well removed from the fluorescence emission (maximum emission, 648 nm).[7] Coherent anti-Stokes Raman scattering (CARS) spectroscopy has been successfully used to obtain RR spectra[8] of phycocyanin with excitation into the intense red absorption band, free from the intense fluorescence that would completely mask the Raman spectrum in a normal RR experiment under these conditions. Inherent in these RR experiments is the requirement for a high optical density of the chromophore in order to obtain satisfactory spectra (e.g., OD 30–50 at 620 nm,[7] OD 5 at 640 nm[8]). More recently, RR spectroscopy has been successfully applied to the Pr form of phytochrome by Fodor et al.[9] They were able to accomplish this by using a sample with an OD of 10 at 668 nm and illuminating it with far-red excitation, thereby avoiding much of the inherent fluorescence of phytochrome.

Surface-enhanced resonance Raman scattering (SERRS) spectroscopy has recently emerged as a method for the analysis of biological materials.[10] Additional enhancement of the resonance Raman spectrum by 10^3 has been predicted due to interaction with the SERRS active surface,[11] thereby increasing the sensitivity of this variant of Raman analysis. More importantly, fluorescence quenching is often observed for the adsorbed species, making possible the observation of the resonance Raman spectrum that would otherwise be completely obscured by an intense fluorescence background. These advantages have allowed us to obtain SERRS spectra of C-phycocyanin chromopeptide isomers and phytochrome[12] using excitation wavelengths that lie in the near-UV absorption band of these compounds. To aid in the interpretation of these spectra, we report here the SERRS analysis of the model compound biliverdin dimethyl ester (BVDE, structure 1).



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Several Raman investigations of BVDE have been reported.[13] Its similar structure to those of the dihydrobilatrienedione chromophores present in phytochrome and the phycocyanins suggests that its spectroscopic properties should depend upon its molecular geometry in a similar manner. However, unlike the situation with proteins, where the conformation of the chromophore is constrained by the surrounding protein matrix, solution studies are limited to analysis of the conformations of BVDE that are the most energetically preferred. Thus, previous resonance Raman investigations have focused upon the analysis of the *Z,Z,Z* isomer of BVDE in the helical conformation, which it preferentially adopts in solution.[4b,13b].

Falk et al.[14] reported the synthesis of the *E,Z,Z* and *Z,Z,E* isomers, formed by irradiation and isomerization of the *Z,Z,Z* isomer while adsorbed on alumina. These isomers are constrained to a geometry different from that of the *Z,Z,Z* isomer and comparison of these different isomers should allow the assessment of the efects of geometry upon their spectroscopic properties. Accordingly, we report the application of SERRS to the analysis of the effects of protonation and isomerization upon the vibrational spectrum of BVDE. Intense, high-quality spectra of the photoisomers were obtained from dilute solutions of the purified compounds. The spectra, which were obtained at low temperature (77 K), are free from photodegradation or isomerization, which affect the analysis at higher temperatures. Significant changes in the spectra are observed upon isomerization and protonation, which have implications regarding the analysis of the phyocyanins and phytochrome.

Experimental Section

A. Materials. Biliverdin dihydrochloride (80% purity) and aluminum oxide (neutral) were obtained from Sigma Chemical Co. BF_3 (50% in methanol) was purchased from Aldrich Chemical Co. Kieselgel 60 Fi54 thin-layer chromatography plates were obtained from Merck, Inc. Na_2SO_4 was purchased from Mallinckrodt Inc. as the reagent-grade material. Biliverdin dihydrochloride used for the synthesis, and all other chemicals were used as received. Water used in the SERRS experiments was distilled first and then purified further by ultraviolet irradiation (Organicpure, Sybron/Barnstead Corp.). Polishing pads and alumina were purchased from Buehler Ltd. (Evanston, IL).

B. Instrumentation. All SERRS spectra were taken by using 413.1-nm laser excitation from a Kr^+ laser (Coherent, INNOVA 100). Laser powers used were typically 5–70 mW at the sample. The sample was irradiated in the backscattering configuration and the scattered radiation was collected and focused onto the entrance slit of a monochromator/spectrograph (Spex, Triplemate 1877). Either 1200 grooves/mm (D^{-1} = 1.4 nm/mm) or 1800 grooves/mm (D^{-1} = 0.9 nm/mm) gratings were used in the spectrograph stage with slit widths of 50–200 μm, corresponding to a spectral band pass of 6–15 cm^{-1}. Two gratings of either 600 or 1200 grooves/mm were used in the monochromator stage. An intensified diode array detector (PARC 1420) coupled to a multichannel analyzer (PARC OMA II) was used to accumulate and process the data.

Potential control during the electrode roughening step was accomplished with a potentiostat constructed in our laboratory.

^1H NMR spectra were acquired on a Varian VXR 200 FT NMR instrument operating at 200 MHz. Spectra were measured in the pulsed and FT mode and are the result of the accumulation of 512 transients. All chemical shifts were referenced with respect to tetramethylsilane by use of the signal from residually protonated acetone-d_6 at 2.04 ppm. Deuterated acetone and chloroform were used as solvents for the *E* and *Z* isomers, respectively.

C. Procedures. 1. Synthesis of BVDE. BVDE was synthesized from biliverdin dihydrochloride after the method of Kufer and Scheer.[15] A 203-mg sample of the dihydrochloride was refluxed for 10 min in 220 mL of boiling methanol containing 8% BF_3. After cooling under N_2 atmosphere, 250 mL of $CHCl_3$ and 250 mL of H_2O were added and the esterified mixture was extracted into the $CHCl_3$ phase. This phase was then separated and washed successively with 250 mL of H_2O, 500 mL of 2 M sodium acetate, and twice more with 250 mL of H_2O. The $CHCl_3$ phase was then filtered by gravity through $CHCl_3$-wetted filter paper. After concentration by removal of much of the solvent by evaporation, the BVDE solution was chromatographed across a column containing 40 g of neutral alumina and eluted with $CHCl_3$. The fast-eluting major band containing the BVDE was collected. All manipulations were performed under very dim green light.

Protonation of BVDE, to form the monoprotonated species, was accomplished by using a method previously reported.[13b]

2. Photoisomerization of BVDE. Neutral alumina (4 g) was added to a 25-mL solution of BVDE (obtained as described above), and the resulting slurry was degassed under vacuum. The solvent was then

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Table I. NMR Peak Assignments

	Z,Z,Z	Z,Z,E	E,ZZ
	Methyl,[a] ppm		
CH_3(2)[b]	1.89	1.90	1.84
CH_3(13)	2.08	2.08	2.08
CH_3(7)	2.10	2.09	2.02
CH_3(17)	2.19	2.11	2.19
	Vinyl,[a] ppm		
=CH(15)	6.03	6.22	
=CH(10)	6.80	6.81	
=CH(5)	6.09	6.05	

[a] Chemical shifts referenced to TMS (see Experimental Section). [b] Ring numbering as shown in structure I (according to Falk et al.,[5b] corrected to follow IUPAC recommendations).

removed by evaporation under vacuum (Rotavapor, 40 °C). Hexane (100 mL) was added to the dried alumina and the N_2-purged slurry was stirred in an ice bath under illumination from a high-intensity tungsten light source (200 W). After 2 h, illumination was discontinued, 25 mL of methanol was added to the flask, and the contents were filtered and washed with methanol. The solvent was removed from the filtrate under vacuum (Rotavapor, 30 °C), and the dried solid was redissolved in a minimum volume of $CHCl_3$. The BVDE isomer mix was then chromatographed over an alumina column (30 g, neutral) with $CHCl_3$ as eluant. The rapidly eluting band containing predominantly the *Z,Z,Z* isomer ("*Z* mix") was collected and the column washed until no further material eluted. The mobile phase was then changed to 2% methanol in $CHCl_3$ and a single eluting band was collected ("*E* mix"). The solutions were stored in amber bottles at −20 °C until further purification prior to use.

3. Purification and Identification of Isomers. The isomer mixtures were purified by thin-layer chromatography on Kieselgel plates (20 × 20 cm, 0.2 mm thickness) using 2% methanol in $CHCl_3$ as the mobile phase. Only a single major spot was observed in the *Z* mix having a relatively high mobility (R_f ca. 1.0). A second minor spot migrated with slightly lower mobility (R_f ca. 0.85), while as many as five very faint components were also detected in this mixture with R_f values ranging from ca. 0.6 to 0.0. The major spot in this mixture was identified as the *Z,Z,Z* isomer by comparison of its absorption, NMR, and Raman spectra with those of the biliverdin dihydrochloride, which were virtually identical. The *E* mix contained three major spots having relatively low mobilities (R_f values ranging from 0.25 to 0.15) as well as faint spots with mobilities that were identical with those of the major and minor spots in the *Z* mix. Irradiation of acetone-eluted solutions of the low-mobility *E*-mix spots indicated that two of the spots were compounds that isomerize to produce the *Z,Z,Z* isomer, from a comparison of the TLC mobilities and of the SERRS spectra of the irradiated solutions. ^{1}H NMR spectra of the two *E*-isomer components allow an identification to be made by comparison of shifts observed in the methyl proton and the vinyl proton regions.[14] The important NMR shifts so obtained are listed in Table I. Using the analysis presented by Falk et al.,[14] we have confirmed the identity of the *Z,Z,E* and *E,Z,Z* isomers by the shifts observed in the resonances of these isomers relative to the *Z,Z,Z* isomer. The identity of the *Z,Z,E* isomer is confirmed by the observed upfield shift of the CH_3(17) of 0.08 ppm (Falk, 0.08 ppm) and the downfield shift in the vinyl C(15)–H resonance of 0.19 ppm (Falk, 0.13 ppm). The identity of the *E,Z,Z* isomer is confirmed by the upfield shift of 0.08 ppm for CH_3(7) (Falk, 0.10 ppm). The poor signal obtained at the low solution concentration used precluded further confirmation using the vinyl resonances of the *E,Z,Z* isomer.

4. Low-Temperature (77 K) SERRS. Silver electrodes were constructed from polycrystalline Ag wire that had been flattened and sealed into glass tubing with a low vapor pressure epoxy resin (Torr-Seal, Varian Assoc.). The exposed, flattened surface (area, 0.1–0.2 cm^2) was polished to a mirrorlike finish by using a sequence of polishing steps with 5.0-, 0.3-, and 0.05-μm alumina. Each polishing step was followed by sonication and rinsing with H_2O. Electrodes polished in this manner yield reproducible roughened surfaces and no sample carryover from one experiment to the next when the same electrode is used.

The SERRS electrode was electrochemically roughened by placing it into a cell containing 0.1 M Na_2SO_4, which had been previously degassed by N_2 purge for at least 15 min. A Pt auxiliary electrode and saturated sodium calomel electrode (SSCE) were used in the cell. The electrode was then cathodized by poising the electrode at a potential of (typically)−2.1 V (vs SSCE) to evolve H_2 as an aid to cleaning the electrode surface and reducing surface oxides that may have formed on the freshly polished surface. Roughening was accomplished (in quiescent solution) by stepping the potential from −600 to +450 mV (vs SSCE) and allowing ca. 25 mC/cm^2 of charge density to pass during the oxidation, whereupon the potential was stepped back to −600 mV until the current decreased to <10 μA/cm^2. This sequence of potential changes is performed automatically by the potentiostat to assure reproducible potential cycling in this step. The electrode surface changes in appearance as a result of this treatment from the mirrorlike silver finish to a gold color, homogeneous in intensity and distributed evenly across the entire electrode surface, which is characteristic of the Ag surface having roughness on a scale of 25–500 nm.[10]

Samples were adsorbed onto the electrode by rinsing the roughened electrode in the appropriate solvent, placing it immediately into the solution, and letting it stand in the dark for 15 min with occasional stirring. The electrode was removed and immediately frozen in liquid N_2. The electrode was then transferred to the Raman Dewar flask containing liquid N_2 and positioned to allow measurement of the SERRS spectrum through the transparent double wall of the Dewar flask while immersed in the liquid N_2.

In most cases, spectra were collected by accumulation of 25 scans (1 scan/s) and stored on floppy disk. Boiling N_2 did not significantly interfere with the accumulation of the spectra when taken in this manner. A background spectrum was occasionally acquired with a roughened electrode on which no sample had been adsorbed. Sample spectra could then be corrected for this background by subtraction. Some of the spectra presented were corrected for background by a polynomial fit of the apparent background with subsequent subtraction. Low-temperature RR spectra were taken by freezing the sample in a glass tube (5-mm o.d.) and placing it into the low-temperature Raman Dewar flask.

Frequency calibration of the spectra was done using indene as a standard. Precision in calibration were observed to be typically ±2 cm^{-1} when using the 1200 grooves/mm grating and ±1 cm^{-1} for the 1800 grooves/mm grating.

Results and Discussion

Surface-enhanced resonance Raman scattering (SERRS) spectra recorded at 77 K were of high quality when the sample was adsorbed from solution concentrations approaching the lower limit practical for conventional resonance Raman methods (10^{-5}–10^{-6} M). The excellent sensitivity of SERRS allows for the direct analysis of the purified eluates from chromatographic separations without preconcentration. Thin-layer chromatography was used in this study to isolate and purify isomers of biliverdin dimethyl ester (BVDE). The SERRS spectrum of the *Z,Z,Z* isomer (Structure I, labeling according to IUPAC recommendations) adsorbed from the acetone-eluted TLC spot is shown in Figure 1A. Resonance Raman spectra observed directly from the same solution under equivalent conditions were generally poor, due to strong solvent bands and a low signal-to-noise ratio.

The excitation wavelength used (413.1 nm, Kr^+) is in resonance with the intense near-UV absorption band (see Figure 1 inset). Low-temperature conditions precluded photodegradation, which occurs with excitation within this region. We observed photodegradation with excitation at room temperature, which was manifest primarily by an increase in the SERRS background and a decrease in the intensity of the SERRS bands. At liquid nitrogen temperatures we observed no significant photodegradation over the period of the measurement (usually <1 min).

The SERRS spectrum of the *Z,Z,Z* isomer eluted in acetone-d_6 (Figure 1B) aids in the assignment of the observed bands. By comparison to results reported by Hsieh and Morris[16] for analysis of bilirubin we may identify lactam-sensitive modes by their rapid exchange with deuterons in the solvent. There is generally good agreement between these deuterium-sensitive bands in BVDE and similar bands reported for bilirubin. Table II lists frequencies and assignments of modes by comparison to bilirubin and normal mode analysis.[13c]

The RR and SERRS spectra of BVDE (*Z* mix; see Experimental Section) are nearly identical (compare Figures 1 and 2). This suggests that resonance of the exciting line with the absorption band dominates the enhancement mechanism under these conditions. No significant mixing of orbitals occurs between the BVDE and Ag surface in the SERRS experiment. This similarity of the SERRS and RR spectra in other systems[17] appears to be

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Table II. Raman Frequencies (cm^{-1})[a]

RR		SERRS					
BR[b]	BVDE	BVDE *Z,Z,Z*-(H)	BVDE *Z,Z,Z*-(D)	BVDE cation	BVDE *Z,ZE*	BVDE *E,Z,Z*	assignments[c]
1630	1631	1635 (sh)?	1635 (sh)?	?	?	?	exo C=C
1615	1619	1618	1614	1615	1616	1616	exo C=C
	1597	1600	1596?	?	1594	1592	
1570	?	1570 (sh)?	?	1576 (sh)	?	?	lactam C=C
1559							pyr C=C
	1539	1535	1525	1549	1535		pyr C=C[d]
1498					1501	1501	lac, pyr[d] C—C
	1472	1467	1467	1462			
1450	1453	?	1450	?			lac C—N, C—C
	1437	1434	1433	1440	1433	1436	lac (C=O)
1436						1418	
	1390	1386	1372	?	1385		
1360	1359	1352	1348	1340	1352		
1341	1331	1325	1324	1329	1325	1362	
1286	1290	1298	1288 (sh)	?	1296	1333	
1268	?	1273 (sh)	1273 (sh)	?		1300	lac ring
	1252	1254	1255	1263	1256	1255	lac ring
1247	?	1245			1247	1243	
1191				1193			lac ring
	1174	1170	1170	1166		1165	ring C—C
1142		1144	1148	1155	1149	1145	
1114		1117		1126	1119	1119	
		1101	1101	?	1096	1096	
		1060	1062	1059	1064	1060	
1050	1050	?		1049	1053	?	
			1022				
990	989	996	992	996	?	?	lac
951	964	966	964	?	?	?	lac (C=O)

[a] From ?, possibly present but unresolved. [b] From ref 16. [c] Based upon solvent sensitivity (ref 16), except where noted. [d] From ref 13c.

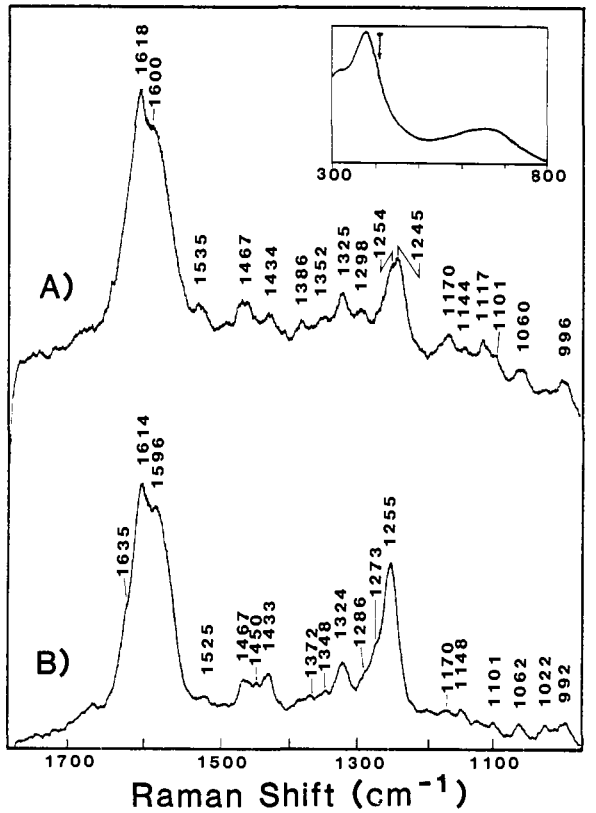

Figure 1. SERRS spectra (77 K) of (*Z,Z,Z*)-BVDE. (A) in acetone (adsorbing solution concentration, $\approx 10^{-5}$ M). (B) in acetone-d_6 (adsorbing solution concentration, $\approx 10^{-5}$ M). Laser power, 70 mW; band pass, 3 cm^{-1}. Inset: UV-vis absorption spectrum; arrow denotes laser excitation wavelength.

a general observation when SERRS enhancement occurs through a predominantly "electromagnetic" rather than a "chemical" enhancement mechanism.[10] Enhancement of the resonance mechanism by as much as 3 orders of magnitude has been predicted by calculations using purely electromagnetic considerations.[11] No attempt has been made to calculate the enhancement factor observed in this experiment, but several observations suggest that a surface enhancement is operative. Small shifts in frequencies are observed in the SERRS spectrum that are indicative of interaction with the Ag surface, the most significant of these being the shift in the band observed at 1331 cm^{-1} in the RR spectrum to 1325 cm^{-1}. Such shifts in frequency are often observed in surface-enhanced Raman spectra[18] and are reflective of the perturbations of vibrational modes upon interaction with the Ag surface. Frequency shifts have been used to deduce the orientations of the adsorbed species with respect to the metal surface;[19]

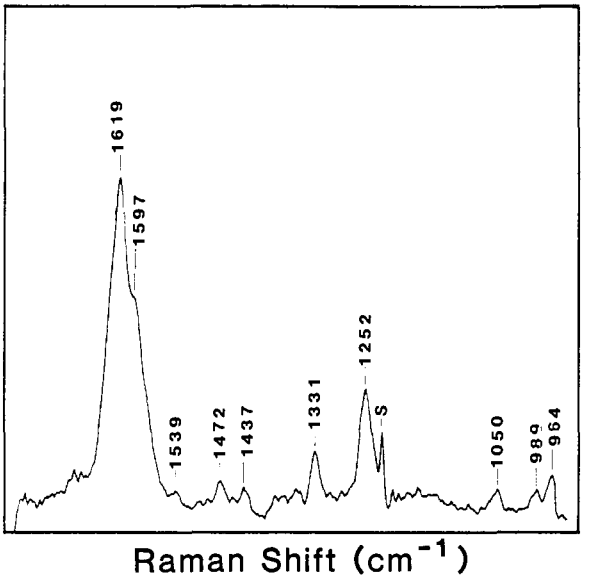

Figure 2. RR spectrum (77 K) of (*Z,Z,Z*)-BVDE (*Z* mix; see Experimental Section). Laser power, 70 mW; band pass, 3 cm^{-1}. Solution concentration, $\approx 10^{-3}$ M. S, solvent peak.

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however, the confident identification of this one band shift only and the absence of definitive assignments make such a deduction tenuous in this case. Additionally, small changes in relative intensity are observed in the SERRS spectrum, which may reflect the sensitivity of the BVDE to the change in its environment upon adsorption to the Ag or, possibly, greater orientational anisotropy in the adsorbed state. Finally, comparison of the RR and SERRS spectra from more dilute solutions (TLC eluates) shows a marked enhancement in the signal-to-noise ratio in the SERRS spectrum. While a surface-enhancement mechanism appears to be operative, a small contribution from conventional resonance Raman enhancement may still be possible as the electrodes were not rinsed to remove nonspecifically adsorbed material prior to the SERRS experiment. Such material may contribute a purely resonance scattering component to the observed spectrum that would be indistinguishable, given the similarity of the SERRS and RR spectra. There is good agreement in the observed frequencies in the SERRS spectra and RR spectra reported by Margulies and Toporowicz[13c] of BVDE in $CHCl_3$ at room temperature using 514.5-nm excitation. Differences in relative intensities are present, particularly with respect to the dominance of the 1300-cm^{-1} band in their spectrum and absence of significant carbonyl bands in the region above 1650 cm^{-1} in our spectra. The intensity profile in our spectra agree better with that reported by Szalontai et al.[7] but differ in the frequency of the band reported at 1268 cm^{-1} in their spectrum. These differences do not arise from interaction with the Ag surface in the SERRS experiment as they are also present in our RR spectrum. The differences may reflect variations in the distribution of conformers present in the solutions in the different experiments. At least three conformationally distinct species have been identified for BVDE *Z,Z,Z* isomer from their different fluorescent lifetimes.[4b,d,e,f] The helical structure is the major component, with more linear conformers comprising a small percentage of the distribution. This distribution is sensitive to environmental influences, which at low concentrations exhibit distinct thermochromic and solvatochromic effects. Protonation in $CHCl_3$ may also be important in producing the observed differences, as is discussed below. The similarity of our RR and SERRS spectra suggests that the major helical component dominates the spectrum in the SERRS experiment. The intensity of the near-UV absorption band, with which we are in resonance, is sensitive to the conformation of the BVDE in solution[5a] and is most intense for the helical conformer. Thus, the spectra arise most likely from the helical structure with little contribution from the more linear conformers in both the RR and SERRS experiments. The absence of the carbonyl band observed at 1691 cm^{-1} with excitation at 514.5 nm[13c] is due to excitation into a different electronic transition in our experiment where the carbonyl modes appear not to be enhanced by resonance. This is supported by the absence of carbonyl modes in the RR spectrum of BVDE[7] when excitation at 363.8 nm is used.

Protonation of BVDE results in a decrease in intensity of bands in the region 1600–1650 cm^{-1}, producing a single sharp band at 1615 cm^{-1} (Figure 3). Other band shifts and changes in relative intensity are observed. The disappearance of the band at 1245 cm^{-1} is significant. This band is one of at least two unresolved bands comprising the intense band whose frequency varies between ca. 1247 and 1255 cm^{-1}, the precise position being determined by the relative intensities of these two bands. The 1245-cm^{-1} band appears to shift upon protonation to produce the more intense 1263-cm^{-1} band in Figure 3. Both bands are sensitive to solvent influences (by analogy to bilirubin results[16]) and the 1245-cm^{-1} band is sensitive to both protonation and deuteration. Consequently, this band has been assigned by Hsieh and Morris[16] to a lactam ring mode. This band will become the focus of interest in the subsequent discussion with respect to SERRS of the BVDE photoisomers.

The protonated BVDE spectrum in Figure 3 differs markedly from that reported by Margulies and Toporowicz.[13c] A RR experiment was performed under the conditions used for the SERRS

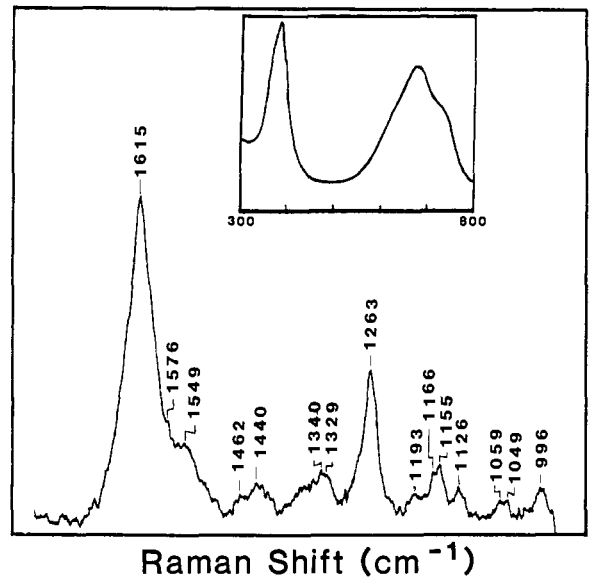
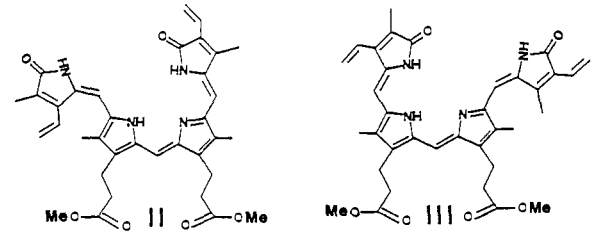


Figure 3. SERRS (77 K) of monoprotonated (*Z,Z,Z*)-BVDE. Laser power, 70 mW; band pass, 3 cm^{-1}. Adsorbing solution concentration, $\approx 10^{-5}$ M. Inset: UV-vis absorption spectrum.

experiment. In the spectrum so obtained we do not observe the strong 1317-cm^{-1} band reported by Margulies and Toporowicz, and the strong 1263-cm^{-1} band remains a major feature, as in the SERRS spectrum. However, these differences might be attributed to the different excitation line that we used (413.1 nm) compared to Margulies and Toporowicz (514.5 nm). On the other hand, the spectrum presented by Szalontai et al.[7] of BVDE in $CHCl_3$ looks remarkably similar to the cation spectrum shown here in Figure 3, particularly with respect to the sharpness of the peaks observed at 1620 and 1268 cm^{-1} and the apparent absence of the 1245-cm^{-1} peak. We have observed the formation of the BVDE cation in dilute solution in $CHCl_3$, possibly arising from protonation by H_2O present in $CHCl_3$. The changes in the BVDE spectrum reported here are similar to changes that are observed in the SERRS spectrum of the cyclic C-phycocyanin chromophore upon protonation[12] and changes reported by Szalontai et al.[7] in the RR spectrum of C-phycocyanin.

Irradiation of BVDE adsorbed on alumina results in photoisomerization to the less stable *E,Z,Z* (structure II) and *Z,Z,E*



(structure III) isomers.[14] Following this method, and upon purification of the compounds by TLC we have obtained well-resolved SERRS spectra (Figure 4). These spectra show qualitative correlations between the SERRS spectrum of BVDE and its geometry. Major differences between the *Z,Z,Z* and *E* isomers include pronounced changes in relative intensities of the bands in the spectra. Of particular interest is the decrease in intensities of the 1245- and 1255-cm^{-1} bands in both of the *E* isomers. The 1245-cm^{-1} band, which is observed in the RR[7a] and CARS[8] spectra of C-phycocyanin, is sensitive to conditions under which the chromophore conformation is observed to change. Although the *E* photoisomers adopt a cyclic conformation,[5b] this conformation is not the helical conformation of the *Z,Z,Z* isomer characterized by a near overlap of rings A and D. This is a consequence of steric hindrance in the *E* isomers that is not present in the *Z,Z,Z* isomer. Normal mode calculations by Margulies and Toporowicz suggest that this 1245-cm^{-1} band may arise from the C(15)–H rocking mode (1237 cm^{-1} calculated value).[13c] By comparison to bilirubin,

(19) Gao, P.; Weaver, M. *J. Phys. Chem.* **1985**, *89*, 5040–5046.

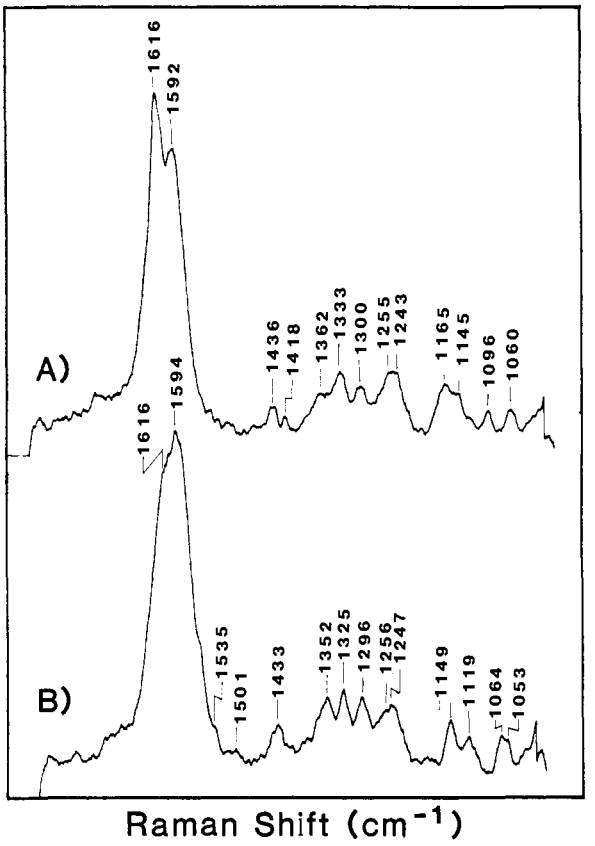
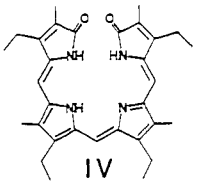


Figure 4. SERRS (77 K) of BVDE photoisomers. (A) (*E*,*Z*,*Z*)-BVDE [adsorbing solution concentration, $\approx 10^{-5}$ M (in acetone)]. (B) (*Z*,*Z*,-*E*)-BVDE [adsorbing solution concentration, $\approx 10^{-5}$ M (in acetone)]. Laser power, 70 mW; band pass, 3 cm^{-1}.

this mode is more likely associated with one of the lactam rings, as identified by its sensitivity to solvent[16] and deuteration (Figure 1). The loss of intensity of this band (and changes in relative intensities of all other bands) can result from at least two sources. First, the isomerization process will produce a change in the normal mode character. It is unlikely that the same internal coordinates will contribute to the individual bands before and following isomerization. Second, band intensities may arise as a consequence of the change in the vibrational overlap integrals upon isomerization. Such a change may be accompanied by a change in the orientation of the electronic transition dipole moment, as a change has been calculated to occur upon isomerization in the similar dihydrobilindione compounds.[5a] Those modes possessing atomic displacement vectors whose components are in the plane of the transition dipole moment will experience larger enhancements than those with components that are perpendicular.[20] The change in orientation of the electronic transition dipole moment that is expected with a change in the molecular geometry (in this instance, photoisomerization) and the concomitant change in the Franck–Condon factors is expected to strongly influence the relative intensities of the bands. Support for this requires confident band assignments, which are still lacking, and a detailed calculation of transition dipole moments for the *Z*,*Z*,*Z* and *E* isomers that is beyond the scope of this work.

The decrease in intensity of the 1245- and 1255-cm^{-1} bands upon isomerization supports the identification of this band as being sensitive to the geometry of the open-chain tetrapyrroles. The results suggest that the increased relative intensity of these bands is a marker for the *Z*,*Z*,*Z* configuration. The sensitivity of these bands to the conformation changes that may occur in the *Z*,*Z*,*Z* isomer to give more linear geometries has not been established for the BVDE. Szalontai et al.[7] has shown that the linear *Z*,*Z*,*Z* chromophore in C-phycocyanin from *Synechococcus 6301* cyclizes to a helical conformation upon lowering of the pH below 3. Concurrent with this cyclization is the observation of a decrease in intensity of the weak band observed at 1245 cm^{-1}. This decrease is likely due to the protonation associated with the lowering of the pH, occurring concurrently with cyclization. Although the comparison of the SERRS results presented here for BVDE with the RR results presented for the C-phycocyanin may not be straightforward, it appears that the sensitivity of this band may be a complicated function of both configuration about the exo double bonds and conformation about the single bonds. Reaction of C-phycocyanin with *p*-(chloromercuri)benzenesulfonate (PCMS) results in modification of a cysteine residue in the vicinity of the chromophore[8] and produces a decrease in intensity of the 1245-cm^{-1} band in the CARS spectrum. This has been interpreted to be evidence for a change in chromophore geometry, possibly a change in the dihedral angle of ring D. This interpretation is consistent with the SERRS results presented for phytochrome.[12c]

Other changes that occur in the SERRS spectrum upon isomerization include the disappearance of the band observed at 1469 cm^{-1} in the *Z*,*Z*,*Z*-isomer spectrum. This band is calculated to be a pyrrole C–C stretch of ring B (1460 cm^{-1}),[13c] and the insensitivity of this band to deuteration observed here is consistent with this assignment. The intensity of this band decreases and shifts to slightly lower frequency upon protonation (Figure 3), which may reflect involvement of the nonprotonated ring. For the *Z*,*Z*,*E* isomer, note especially the changes in the relative intensities and small frequency shifts of the major bands that are observed in the C=C envelope between 1550 and 1650 cm^{-1}, and the dramatic increase in intensity of the 1594-cm^{-1} band. The increase in intensity of this 1594-cm^{-1} band seems to be a good indicator of a *Z*,*Z*,*Z* to *Z*,*Z*,*E* isomerization and allows for the differentiation between *E*,*Z*,*Z* and *Z*,*Z*,*E* isomers. No other significant band shifts are observed in the spectrum of the *Z*,*Z*,*E* isomer, while a number of other band shifts (1325 to 1333 cm^{-1}, 1170 to 1165 cm^{-1}) are observed upon isomerization to the *E*,*Z*,*Z* isomer. These shifts in frequency may be associated with an increase in the torsional angle predicted at the methine single bond when isomerization occurs at the corresponding double bond. For the closely related alkyl-substituted tetrapyrrole (structure IV),



IV

this angle increases from 20° in the *Z*,*Z*,*Z* isomer to 40° (the maximum possible without disruption of the π subsystem[5a]) in the *Z*,*Z*,*E* isomer.[5b] Differences in the spectra of the *E*,*Z*,*Z* and *Z*,*Z*,*E* isomers may then be due to a greater out-of-helix twist of the isomerized ring A in the *E*,*Z*,*Z* isomer. This greater twist is produced by greater steric hindrance of the peripheral substituents at positions C(3) and C(7) as compared to C(13) and C(17) in the *Z*,*Z*,*E* isomer (compare structures II and III). The fractional change in bond order resulting from this twisting may then produce the frequency shifts that we observe upon photoisomerization. This same twisting of rings A or D may also be responsible for the changes in relative intensities of the 1245- and 1255-cm^{-1} bands.

The dissimilarity of the *E*-isomer spectra and *Z*,*Z*,*Z*-isomer spectrum provides evidence for the stabilization of the BVDE on the Ag surface to photoisomerization during the SERRS experiment. The photoisomerization of these *E* isomers to the more stable *Z*,*Z*,*Z* isomer occurs readily upon illumination with white light. No significant formation of this isomer occurs as a result of laser illumination during the experiment as the 1469-cm^{-1} band does not appear nor are the other corresponding changes observed to occur.

Conclusions

Low-temperature (77 K) SERRS provides intense, well-resolved resonance Raman spectra of BVDE from dilute solutions. These

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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.

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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 $Z,Z,Z \rightarrow Z,Z,E$ 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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