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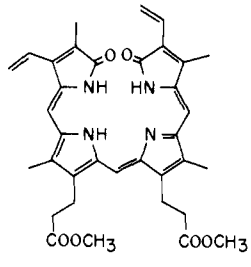
Spectroscopic Studies on Model Compounds of the Phytochrome Chromophore. Protonation and Deprotonation of Biliverdin Dimethyl Ester

Sir:

Phytochrome (P) is the pigment responsible for many photomorphogenic responses in plants.[1] It is a chromoprotein which is known to exist in two different forms, interconvertible by light: a red absorbing form (P_r, λ_{max} 667 nm) which is thermodynamically stable and physiologically inactive, and far-red absorbing form (P_{fr}, λ_{max} 730 nm) which is the physiologically active form of the pigment.[2,3] The chromophore of P has been found to have a bilitriene structure, but the structural differences between P_r and P_{fr} is still unclear. Various models have been suggested for explaining the phototransformation[4-13] and the importance of charged chromophoric structures has been recognized.[7,12]

We have started a systematic study of the spectral and conformational properties of model compounds for the chromophore of P aiming at a deeper insight into the mechanisms of action of the natural pigment. We report herein a spectroscopic study of the protonation and deprotonation of biliverdin dimethyl ester (**1**), a bile pigment which apparently has



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structure and absorption properties similar to those of the chromophore of P.[8,9,11] For the first time we present evidence, particularly obtained from resonance Raman experiments, on the presence of different protonated species of **1** in media of different acidities.

Compound **1** was synthetized from bilirubin and purified by recrystallization and column chromatography. The same results were obtained with a pure sample which was prepared in a different way by another group.[13] Chloroform was chromatographied on basic alumina before use. All solutions were oxygen free and were handled under N_2 and diffuse light. Proton concentration was controlled by shaking the chloroform solution of **1** with aqueous HCl of known concentration.

The pH dependence of the absorption spectrum is shown in Figure 1.

The absorption spectrum of **1** in neutral chloroform shows a broad band centered at 660 nm ("red" band), a strong band at 379 nm ("blue" band), and two small peaks at 317 and 280 nm (Figure 1, top). In mildly acidic solutions (shaking with 1 N HCl) major changes are observed in the red band (Figure 1, middle): the maximum is shifted to 674 nm, a shoulder is observed at 723 nm, and the overall intensity is increased by

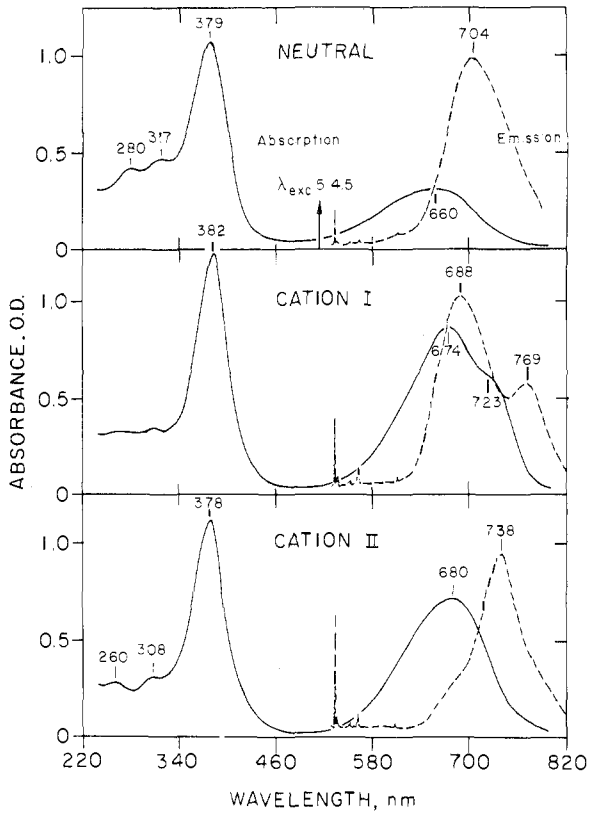

Figure 1. Absorption and emission spectra of **1** in chloroform. Notation on the ordinate refers to absorption spectra only; emission spectra (uncorrected) are given in relative units.

a factor of 2.5. The blue band shifts to 382 nm and the transitions at shorter wavelengths are smoothed out. In strong acidic solutions (shaking with concentrated HCl) a different absorption spectrum is observed (Figure 1, bottom). The red band slightly decreases in intensity, loses its structure, and shifts to 680 nm. The blue band shifts to the blue (378 nm) and two bands are observed at 308 and 260 nm. We shall refer to the species responsible for the absorption spectra observed in weak and strong acidic solutions as cation I and cation II, respectively.

Resonance Raman (RR) spectroscopy is a method which has already been successfully used to study chromophoric groups in photobiological systems.[14,15] The different identity of cations I and II is more evident from the RR spectra shown in Figure 2. The spectra presented here were measured using the 514.5-nm Ar^+ laser line for excitation, and represent the first report on the applicability of the RR technique to the study of bile pigments.[16]

The RR spectrum is drastically changed when passing from a neutral solution of **1** to cation I. Among other changes, the disappearance of vibrational lines at 1244, 1300, and 1435 cm^{-1}, the appearance of a strong band at 1317 cm^{-1}, and the shifts and intensity changes observed around 1600 and 1700 cm^{-1} are remarkable.

The RR spectrum of cation II is completely different from that of the neutral compound as well as from cation I. New vibrational lines appear at 1153, 1267, and 1325 cm^{-1}, two strong lines of comparable intensity can be observed at 1619 and 1631 cm^{-1}, and the vibration around 1700 cm^{-1} disappears.

Further evidence for the different character of cations I and II is extracted from the fluorescence spectra shown in Figure 1. These spectra were recorded on the Raman spectrometer. The high sensitivity of the detection system of the instrument allows the measurement of the emission spectra of **1** at room

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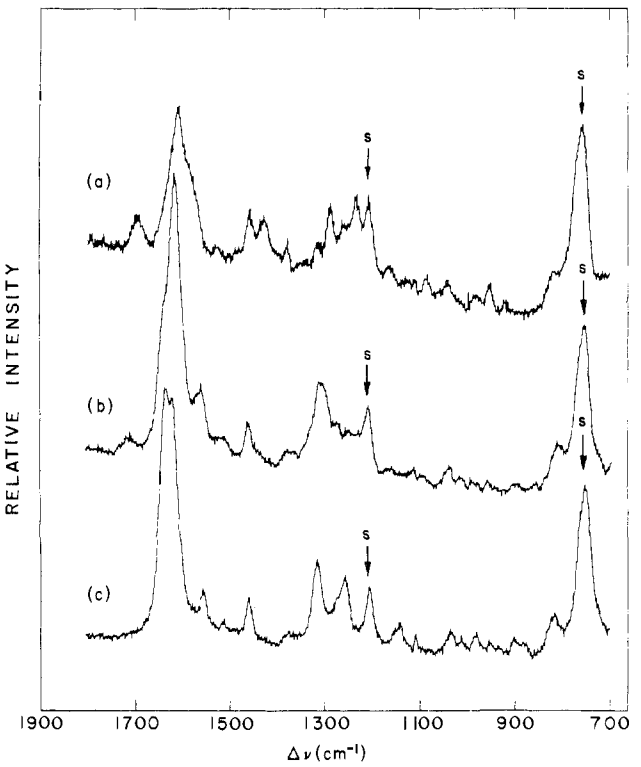

Figure 2. Resonance Raman spectra of **1** in chloroform: (a) neutral; (b) cation I; (c) cation II. Solvent lines are indicated by arrows.

temperature, despite the very low fluorescence quantum yield reported.[17] The Raman spectra of the solutions can be observed on the blue side of the fluorescence bands. Though the spectra are uncorrected for the instrumental sensitivity, some qualitative conclusions may be drawn. The neutral chloroform solution of **1** shows a broad fluorescence band with a maximum at ~704 nm which is split into two maxima (688 and 769 nm) upon shaking with 1 N HCl (cation I). Cation II has a maximum fluorescence at 738 nm, although a shoulder is still present at ~690 nm.[18]

It may therefore be concluded from the experimental evidence presented that different protonated species are present in media of different acidities.

The protonation effects are reversible. By shaking a solution containing cations I or II with 1 N aqueous NaOH, the original "neutral" absorption, fluorescence and RR spectra are recovered. This proves that we are dealing here with a pure protonation–protonation effect, and that the species identified as cations I and II do not correspond to degradation products of **1**.

A complete RR study of **1**, covering polarization and excitation profile measurements and a theoretical conformational analysis, is still in progress.

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On the Origin of the Red Shift of the Absorption Spectra of Aggregated Chlorophylls

Sir:

The reaction center of photosystem I in green plants (P700) is believed to involve a special dimer of a monohydrated chlorophyll *a* (Chl $a \cdot H_2O)_2$ (for recent reviews see ref 1–3 and references cited therein). Understanding the electronic potential surfaces of this dimer is essential for the elucidation of the dynamics and mechanism of the first step of the photosynthetic process. Direct information about the electronic states of chlorophyll polymers come from the absorption spectra of different types of aggregated chlorphylls. While the monomeric hydrated Chl *a* absorbs at ~665 nm (15 037 cm^{-1}),[2] the photoreactive dimer (P700) and related in vitro prepared dimers absorb at ~700 nm (14 286 cm^{-1}). Crystals and some types of surface layers absorb near 735 nm (13 605 cm^{-1}).[4]

The nature of the absorption red shift observed in Chl *a* aggregates has not been resolved despite many studies.[2-7] It was realized in recent years[2-4,7] that the red shift cannot be due to simple exciton interactions, because these account for only about 300 cm^{-1} of the observed shift. Shipman et al.[3b] and Fong and Wassam[2] attributed the large difference between the calculated exciton shift and the observed shift to environmental effects (dipole–dipole interactions with the solvent and/or other chlorophyll molecules). Kartky and Dunitz[4] suggested that the major inadequacy of the exciton model is the breakdown of the zero overlap approximation. Unfortunately, these explanations were not verified by calculations or by direct experiments and the origin of the dimer red shift remains obscure.

As long as the structure of P700 is not known, it is impossible to determine definitely the origin of its red shift. However, now it is possible to calculate the absorption spectrum of a chlorophyll dimer in a nonpolar solvent and to find out whether it can account for the red shift of P700. Here we present such quantum mechanical calculation on a chlorophyll dimer, where the two molecules are fixed in the relative geometry found in crystals of chlorophyll derivatives and surrounded by a nonpolar solvent. *This calculation gives a red shift almost as great as that found in P700.* The same type of calculation also reproduces the experimental red shift in a well-defined system of a crystal of a chlorophyll derivative. Our calculations employ the QCFF/PI method[8] which was used previously to evaluate geometries, spectra, and vibronic intensities of excimers in

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