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RESONANCE RAMAN SPECTROELECTROCHEMISTRY OF BACTERIOCHLOROPHYLL AND BACTERIOCHLOROPHYLL CATION RADICAL

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SUMMARY. The newly developed technique of resonance Raman spectroelectrochemistry (RRSE) is applied to the study of the radical ion species involved in the primary photochemistry of photosynthesis. By means of controlled potential coulometry combined with resonance Raman spectroscopic detection, the vibrational spectrum of the bacteriochlorophyll a cation radical ($BChl^{+\cdot}$) has been obtained and compared with the corresponding spectrum of the parent molecule. The cation radical spectrum is significantly different from the neutral spectrum both in band frequencies and intensities. These results suggest that RR vibrational spectra may provide a new means of identifying and kinetically monitoring radical ion formation both in photosynthetic model systems and in vivo.

INTRODUCTION. Resonance Raman spectroscopy (RRS) is now widely recognized as a highly sensitive and selective probe of molecular and electronic structure in biological molecules. Extensive investigations of heme proteins (1-6), visual pigments (6,7), and metalloporphyrin model systems by RRS (6,8) have been reported. In addition Lutz has recently employed RRS for studying the interactions between various chlorophylls and their environments in photosynthetic systems (9-11).

Another aspect of photosynthesis which should be amenable to direct investigation by RRS is the identification and kinetic monitoring of radical ion formation. Radical ions play an essential role in the process of photo-induced charge separation in photosynthesis. In particular, five different radical ion intermediates have been observed in photosynthetic bacterial reaction centers following light excitation of the primary electron donor, bacteriochlorophyll (BChl). These include: 1) the dimer cation radical of the donor, $(BChl)_2^{+\cdot}$, (12-14); 2) the anion radical of the intermediate electron

Abbreviations: RRS: resonance Raman spectroscopy; BChl: bacteriochlorophyll a; BPh: bacteriopheophytin a; UQ: ubiquinone; RRSE: resonance Raman spectroelectrochemistry; TCNE: tetracyanoethylene; TCNQ: tetracyanoquinodimethane; TMPD: tetramethyl-p-phenylenediamine; TTF: tetrathiafulvalene; R. : Rhodopseudomonas; TBAP: tetrabutylammonium perchlorate; PtQRE: platinum quasi-reference electrode; SCE: standard calomel electrode; CW: continuous wave; Rh110: Rhodamine 110; Rh6G: Rhodamine 6G; ϵ = molar extinction coefficient.

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acceptor, bacteriopheophytin (BPh^-) (15,16); 3) ubiquinone anion radical (UQ^-) (17,18); 4) UQ^--nonheme iron complex (19-21); and 5) $P800^-$, which may be either $BChl^-$ or another BPh^- (22,23). The identification of these radical ions *in vivo* has been accomplished by comparing their visible absorption, esr, and endor spectroscopic properties with those of radical ions generated *in vitro* by electrochemical (15,16,24-26) and pulse radiolysis (27) techniques.

In this communication we report the first of a series of studies aimed at the *in vitro* characterization of photosynthetically important radical ions by the newly developed and powerful technique of resonance Raman spectroelectrochemistry (RRSE). This hybrid technique, combining RRS detection with various electrochemical radical ion generation procedures, has already proven to be a sensitive technique with high molecular specificity for the identification and electronic structure characterization of the anion radicals of tetracyanoethylene ($TCNE^{\cdot}$) (28), tetracyanoquinodimethane ($TCNQ^{\cdot}$) (29,30), and the cation radicals of tetramethyl-p-phenylenediamine ($TMPD^{\cdot+}$) (31) and tetrathiafulvalene ($TTF^{\cdot+}$) (32,33). The objectives of the present study were to demonstrate that room temperature, solution RR spectra of BChl and $BChl^{\cdot+}$, as compared to the low temperature (35°K), aggregated film BChl spectra of Lutz, *et al.* (10), could in fact be obtained and to determine which vibrational features, if any, would serve to identify $BChl^{\cdot+}$ in the presence of BChl. We anticipated that the vibrational frequency shifts induced by radical ion formation would be large enough to permit unambiguous detection and identification of radical ion intermediates both in photoinduced electron transfer model systems and in photosynthetic bacteria.

MATERIALS AND METHODS. BChl was extracted from *R. spheroides* and purified by sucrose column chromatography (34). Exposure to light and oxygen was minimized during isolation and purified BChl was stored under vacuum and at 0°C. CH_2Cl_2 was dried over 3Å molecular sieves and vacuum distilled for preparation of the neutral BChl solutions. The BChl solutions were degassed under diffusion pump vacuum by repeated freeze-pump-thaw cycles and sealed in 5 mm o.d. Pyrex tubes for RR spectroscopy. The visible absorption spectra of the solutions were recorded before and after RRS to check for laser induced photodecomposition of the BChl and none was found.

$BChl^{\cdot+}$ was prepared by exhaustive electrooxidation at a platinum foil working electrode in vacuum degassed CH_2Cl_2 containing 0.1 M tetrabutylammonium perchlorate (TBAP) supporting electrolyte. The purification procedure for TBAP has been previously described (28). In order to avoid any possibility of H_2O contamination in the electrogeneration procedure for $BChl^{\cdot+}$, a platinum quasi-reference electrode (PtQRE) rather than an SCE was used. The working electrode potential for quantitatively converting BChl to $BChl^{\cdot+}$ was determined by cyclic voltammetry on the BChl solution just prior to the bulk electrolysis. Typically, the electrooxidation of BChl was carried out at +0.35 volts vs PtQRE which is more than 60 millivolts positive of E_p for $BChl \rightleftharpoons BChl^{\cdot+} + e^-$. At this potential electrogeneration of $BChl^{\cdot+}$ was quantitative as determined from coulometry and optical

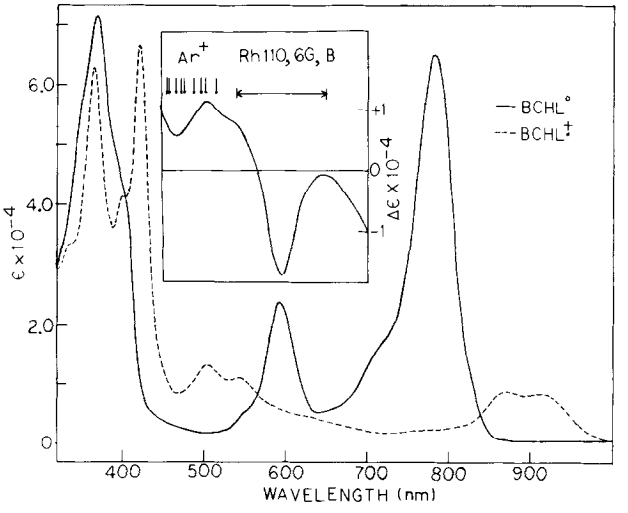

Figure 1. Electronic absorption spectra of BChl and $BChl^{+\cdot}$ from 300-1000 nm. (——) BChl, 8 x 10^{-4} M in CH_2Cl_2. (---) $BChl^{+}$, 8 x 10^{-4} M in CH_2Cl_2 containing 0.1 M TBAP. Cell pathlength = 0.10 mm. Insert is the difference spectrum for $BChl^{+}$ minus BChl neutral. Arrows indicate position of laser excitation wavelengths on nm scale. The abscissa of insert is identical to that of absorption spectrum.

spectroscopy. $BChl^{+}$ was shown to be extremely stable in the electrogeneration medium. Reversal coulometry after completion of the RR scan resulted in greater than 95% recovery of BChl. Further details concerning the electrochemistry of BChl and the vacuum electrolysis cells for these RRSE experiments will be published elsewhere (35).

RR spectra for both BChl and $BChl^{+}$ were obtained by excitation with various lines from a Coherent Radiation Laboratories Model CR-3 argon ion laser. In addition RR spectra for BChl were obtained using an argon ion pumped CW dye laser. Plasma lines from the ion laser and superradiant fluorescence from the CW dye laser were removed with a Littrow prism premonochromator. To avoid decomposition at the laser beam focus, the sample was either spun or stirred rapidly to move the sample with respect to the beam. The Raman scattered light was collected with an f 1.6 camera lens in backscattering geometry and focused onto the slits of a 0.75 meter Spex Model 1400-II double monochromator equipped with a cooled RCA C31034A photomultiplier tube and standard low level threshold photon counting detection electronics. The RR data were collected and processed on-line with a Raytheon 500 minicomputer system interfaced to the Raman spectrometer. The computer system is equipped with disc memory, magnetic tape storage, storage display oscilloscope, digital incremental plotter, and line printer. The RR spectrometer was wavelength calibrated by means of Ar^{+} laser plasma lines and the vibrational frequencies reported here are believed to be accurate to ± 2 cm^{-1}. Depolarization ratios were measured using a polaroid analyzer and a polarization scrambler.

RESULTS AND DISCUSSION.

Electronic Absorption Spectroscopy of BChl Neutral and $BChl^{+\cdot}$

The electronic absorption spectrum of a 10^{-3} M solution of neutral BChl in CH_2Cl_2 is shown in Figure 1 (solid line). The assignment of the perpendicularly polarized, in-plane transitions Q_Y (770nm) and Q_X (583nm) is well established from fluorescence polarization studies (36). On the other hand, the Soret region of the absorption spectrum is complex and is known to contain at least five transitions (37), the most intense of which are the B_x (391nm) and the B_y (358nm). Theoretical analyses have predicted an x-polarized transition on the red shoulder of the B_x band (37,38) and a definite shoulder is observed experimentally (Figure 1).

Controlled potential bulk electrolysis of the 10^{-3} M solution of BChl in CH_2Cl_2 at +0.35 volts vs PtQRE results in the one electron oxidation of BChl. The absorption spectrum of the cation radical is shown in Figure 1 (dashed line) and is identical with that of Fajer et al. (24). Removal of an electron from the highest occupied molecular orbital of the neutral BChl molecule causes a substantial red shift of the Q_y and B_x transitions. In addition two bands are observed at 505nm and 545nm. These transitions have not been assigned, but the molecular orbital calculations of Otten predict weak transitions in this region (38).

The insert in Figure 1 illustrates the difference spectrum of $BChl^{+\cdot}$ minus BChl neutral for the wavelength region spanned by the available CW laser sources. In order to optimize conditions for observing the RR spectrum of BChl neutral, dye laser excitation (580nm) is indicated since the extinction coefficient of BChl neutral is greatest in this region (a minimum occurs in the difference spectrum). On the other hand, a preliminary inspection of the difference spectrum suggests that excitation with the 501.7 or 514.5nm lines of the Ar^+ laser should be near optimal for obtaining the RR spectrum of $BChl^{+\cdot}$.

Resonance Raman Scattering Spectroscopy of Bacteriochlorophyll Neutral.

Lutz and co-workers have reported the RR spectrum of a BChl film at 35°K obtained by excitation at 579nm or in the Q_x transition (10). We have successfully obtained spectra of BChl in solution and at room temperature throughout the excitation range spanned by the Rhodamine 6G CW dye laser (580-620nm). The spectra obtained by excitation near (580nm) are similar to those of Lutz et al. In addition, however, the intensities of several of the vibrational modes were found to be markedly dependent upon the excitation frequency in this region. Further details of these RR spectra will be published elsewhere.

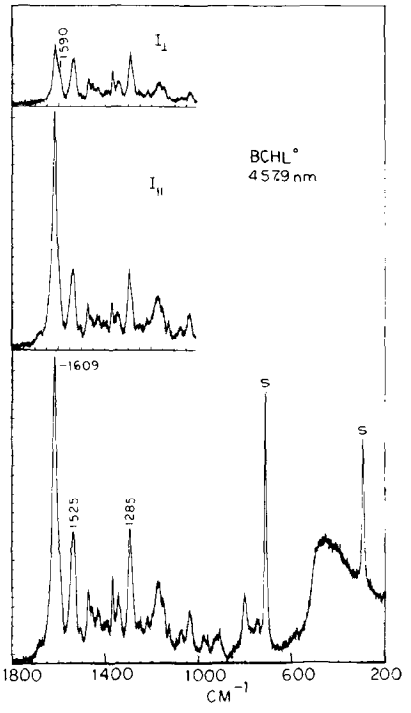

Figure 2. Resonance Raman spectrum of 1 x 10^{-2} M BChl solution in CH_2Cl_2 at room temperature. Excitation wavelength = 457.9 nm; laser power = 40 mw, bandpass = 3 cm^{-1} sec^{-1}, scan rate = 0.167 cm^{-1} sec^{-1} and counting interval = 1.0 second. S refers to solvent peaks. Upper and middle spectra are for scattered radiation polarized perpendicular ($I_\perp$) and parallel ($I_\parallel$) respectively to the polarization of the incident radiation.

The use of Ar^+ laser lines to obtain RR spectra of neutral BChl solutions results in spectra which are quite different from those obtained by excitation into the Q_x transition. Our results indicate that the enhancement of the vibrational spectra is a result of coupling to the Soret electronic transitions. Excitation at 514.5nm yields very weak RR spectra since the molar extinction coefficient is small at this wavelength (BChl ϵ(514.5nm) = 2400 $M^{-1}Cm^{-1}$). As the excitation wavelength approaches the Soret transitions, the RR spectra become more intense. Excitation at 457.9nm, which is in the red tail of the intense Soret transitions, produces spectra with acceptable signal to noise ratios. Figure 2 illustrates the RR spectrum of a 10^{-2}M solution of BChl in CH_2Cl_2. The lower spectrum is that obtained for unpolarized scattered light, while the upper and middle spectra are for scattered light which is polarized perpendicular and parallel respectively to the incident polarization. Unlike the RR spectrum obtained by excitation

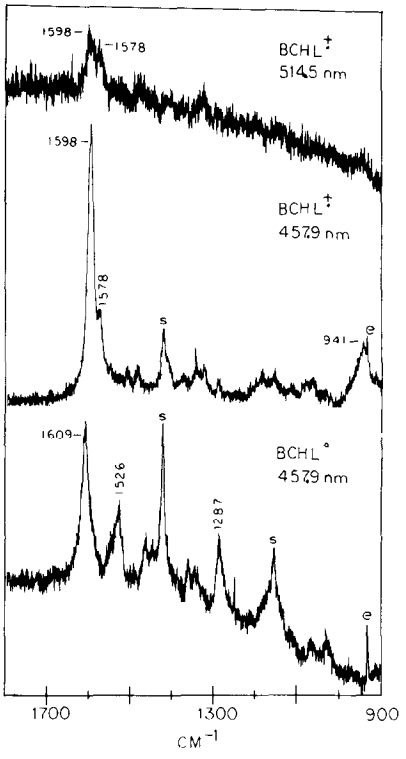

Figure 3. Resonance Raman spectra of a 1.3×10^{-3} M solution of BChl cation radical in CH_2Cl_2 + 0.1 M TBAP obtained by excitation at 514.5 nm (upper spectrum) and 457.9 nm (middle spectrum). The lower spectrum is for an identical concentration of BChl neutral in CH_2Cl_2 obtained by excitation at 457.9 nm. $BChl^+$ was electrogenerated at +0.35 volts vs PtQRE. Spectra were recorded at 30 mw power, 3 cm^{-1} bandpass, scan rate = 0.167 cm^{-1} sec^{-1} and a 1.0 sec counting interval. S refers to solvent bands; e is an electrolyte band (ClO_4^-).

into the Q_x transition, the low frequency vibrations (below 1000 cm^{-1}) are relatively weak. The most intense feature is the 1609 cm^{-1} band which has been assigned to the methine C=C stretch (10). This vibration is totally symmetric (ρ = 0.28). The next most intense bands are at 1528 cm^{-1} and 1285 cm^{-1} and both have polarization ratios near 0.7, which is close to the value for non-totally symmetric vibrations. These polarization results are unusual in that excitation in the Soret band of porphyrins usually results in resonance enhancement of only totally symmetric vibrations (6). Possible explanations for these results are that excitation of BChl at 457.9nm involves a transition with charge transfer character or that mixing of excited states is occurring. The RR spectrum obtained by excitation at

457.9nm, while not a definite proof of, is consistent with an x-polarized transition in this region. The C=O vibrational modes (C-2 acetyl and C-9 carbonyl) are not resonance enhanced and should not be on the basis of Hirakawa and Tsuboi's Rule (39) if the electronic transition is along the x-axis of the molecule. Since the Soret region of the BChl spectrum is complex, a better understanding of the electronic transitions and the coupled vibrational spectra will require detailed excitation spectra in the region between 380 and 450nm.

Although the molar extinction coefficient of BChl neutral at 457.9nm is only $3000M^{-1}Cm^{-1}$, RR spectra with adequate signal to noise may be obtained at BChl concentrations as low as 5×10^{-4} M. The RR spectrum of a 10^{-3} M solution in CH_2Cl_2 obtained by excitation at 457.9nm is shown in Figure 3. Although many of the weaker bands are obscured by the background signal, the stronger bands are identical in relative intensity and frequency to those in the spectrum of the more concentrated solution (Figure 2). In any case, careful sample preparation is essential to eliminate fluorescent impurities and to prevent laser induced photodegradation of the BChl.

Resonance Raman Scattering Spectroscopy of $BChl^{+\cdot}$

The BChl cation radical was electrogenerated in CH_2Cl_2 solutions at concentrations of 10^{-3}M or less to avoid possible dimerization and/or disproportionation reactions. Figure 3 shows the RR spectrum of $BChl^{+\cdot}$ obtained by excitation at 514.5nm (top spectrum). Surprisingly, excitation at this wavelength or 501.7nm, both of which are near the 505nm absorption band of $BChl^{+\cdot}$ did not result in very intense RR spectra. Yet intense RR spectra of $TCNQ^{-\cdot}$ have been obtained at a similar concentration even though the molar extinction coefficient is only 1/3 that of BChl at 514.5nm (29,30). The weak spectra obtained for $BChl^{+\cdot}$ for excitations near the 505nm absorption may be due to interference effects (40). The results also underscore the fact that vibrational intensities in RR spectroscopy are not governed by the molar extinction coefficient alone but are affected by the Franck-Condon factors and the excited state linewidth, Γ, of the electronic transition (33). If interference effects are not responsible for the weak intensities obtained by 514.5nm excitation, it may be concluded that the Franck-Condon factors (i.e., bond length and frequency changes) are small or that the excited state linewidths are broad for the 505nm transition in $BChl^{+\cdot}$.

Since excitation at 514.5 or 501.7nm did not yield very intense RR spectra, the next logical excitation wavelength to use was 457.9nm. The molar extinction coefficient at this wavelength is only slightly less than that at either of the previously discussed wavelengths. However, referring

to Figure 1, it may be noted that 457.9nm is near the foot of the 420nm (B_x) transition in the $BChl^{+\cdot}$ absorption spectrum. The 420nm absorption band in $BChl^{+\cdot}$ is a different electronic transition and the three variables governing band intensities are also expected to be different from those for the 505nm transition. Indeed, a strong RR spectrum was obtained by excitation at 457.9nm as seen in Figure 3. A comparison of the spectrum of the cation radical with that of the neutral species at the same concentration indicates the changes which occur in the RR spectrum on one electron oxidation of BChl. The 1609 cm^{-1} band of the neutral molecule is intensified by a factor of approximately 3 and shifted to 1598 cm^{-1}, or 11 cm^{-1} to lower frequency. The polarization ratio of this band is 0.25, again indicating a totally symmetric vibration. The vibrations at 1528 and 1286 cm^{-1} are reduced in intensity and are not readily assignable at this concentration. Only small peaks are observed at lower frequencies than those of the neutral molecule. In addition, a new broad vibration is observed near 941 cm^{-1}.

The changes which have been described above in the RR spectrum of BChl upon oxidation probably result from π bond order changes in the bonds involved in these vibrations. Jeanmaire and Van Duyne (29) have correlated the large TCNQ vibrational frequency shifts upon formation of $TCNQ^{-\cdot}$ with bond order changes. In the case of BChl much smaller bond order changes may account for the smaller frequency shifts. Another factor which should be considered in accounting for the intensification of the methine C=C stretching vibration on oxidation of BChl is the improved resonance with the B_x transition. Since the B_x transition is red-shifted to 420nm in the cation radical, excitation at 457.9nm is much closer to resonance with this transition in the cation radical than in the neutral molecule. In general, improved resonance with the Soret transitions is known to enhance totally symmetric vibrations in RR spectroscopy (41). Although no RR spectra have been previously reported for metalloporphyrin cation radicals, the RR spectra of metalloporphyrin anion radicals have, in some cases, shown a relative weakening of non-totally symmetric vibrations as the excitation wavelength approaches the Soret transitons (42). Clearly, RR excitation spectroscopy is needed in the Soret region for $BChl^{+\cdot}$ as well as BChl neutral.

In conclusion, the changes which we have observed in the RR spectrum of BChl upon oxidation to the cation radical are significant. An understanding of these changes awaits further experimentation, expecially excitation in those regions of the absorption spectrum which are closer to exact resonance with the Soret transitions. However, the results are encouraging in that RRSE is capable of differentiating between neutral BChl and $BChl^{+\cdot}$. Charac-

terization of the radical ions of BPh and the anion radicals of BChl and UQ is in progress. It is anticipated that RRSE may provide a new and unambiguous technique for identifying radical ions in photosynthetic systems.

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