Observation of the Resonance Raman Spectra of the Semiquinones $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ in Photosynthetic Reaction Centers from *Rhodobacter sphaeroides* R26

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Received October 11, 1996
Revised Manuscript Received April 8, 1997

The two quinone molecules (Q_A and Q_B) in photosynthetic reaction center protein complexes (RCs) of purple bacteria play essential roles in the conversion of light energy into chemical energy.[1,2] While both Q_A and Q_B are ubiquinone (Q_{10}) in *Rhodobacter sphaeroides* RCs, Q_A acts as a one electron carrier only, whereas Q_B accepts two electrons and two protons before leaving the RC site as a dihydroquinone (Q_BH_2).[3] The structure-function relationship of Q_A and Q_B has been a matter of concern and interest of X-ray,[4−6] EPR,[7] ENDOR,[8] and IR studies.[9,10] Resonance Raman (RR) spectroscopy is expected to give detailed information on the C═O and C═C bonds of quinone anion radicals similar to the studies *in vitro*,[11−14] but so far no RR spectra have been reported for $Q_A^{\bullet-}$ or $Q_B^{\bullet-}$ of RCs. In this study, we succeeded in selectively observing the RR spectra of $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ in *Rb. sphaeroides* RCs for the first time and confirmed it by ^{13}C-isotopic frequency shifts. The spectral differences between $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ are interpreted on the basis of the solvent effects of RR spectra of $Q_{10}^{\bullet-}$.

RCs from *Rb. sphaeroides* (R26) were purified in LDAO (lauryldimethylamine *N*-oxide) as described.[15] RC samples containing one quinone/RC (Q_A RCs) were prepared through removal of Q_B by incubation in LDAO and *o*-phenanthroline.[16] Samples containing 3−5 quinones/RC (Q_B RCs) were prepared by adding additional quinones to purified RCs samples. ^{13}C-Q_{10} was obtained from *Rb. sphaeroides* grown using ^{13}C-acetate as the sole carbon source.[10] The ^{13}C-Q_{10} was incorporated into the RCs by incubation of RCs containing Q_A with ^{13}C-Q_{10} over a period of 2 days (4 °C, 4 Q_{10}/RC). Under these conditions Q_B was fully exchanged for ^{13}C-Q_{10}, but Q_A was ∼50% exchanged.

Q_{10} (Sigma) was used without further purification and $Q_{10}^{\bullet-}$ was prepared by electrolysis in organic solvents using the cell specifically designed for the Raman and absorption measurements.[13] Raman scattering was excited at 441.6 nm near the absorption maximum of an isolated $Q_{10}^{\bullet-}$ using a He-Cd laser (Kimmon Electronic, CD4805R). The scattered light at 135° in the back scattering geometry was passed through a holographic filter and dispersed with a 25 cm spectrograph (Chromex 250IS) equipped with a CCD detector (PAR, OMA 4). The RR spectra of ^{12}C-$Q_A^{\bullet-}$ and ^{12}C-$Q_B^{\bullet-}$ were obtained from the RCs containing only Q_A or both Q_A and Q_B, that were illuminated (5 mW at 590 nm) to give the charge separated states, $D^+Q_A^-$ and $D^+Q_B^-$, respectively. The contributions from the oxidized bacteriochlorophyll dimer and other pigments were removed by subtracting the RR spectrum of RCs chemically oxidized by addition of 8 mM potassium ferricyanide. RR spectral intensities of neutral Q_A and Q_B can be neglected in the present experimental conditions.[14]

Figure 1(a) shows RR spectra of ^{12}C-$Q_A^{\bullet-}$ (A), ^{13}C-$Q_A^{\bullet-}$ (B), ^{12}C-$Q_B^{\bullet-}$ (C), and ^{13}C-$Q_B^{\bullet-}$ (D) in RCs. Three bands have been observed at 1605, 1523, and 1486 cm^{-1} for $Q_A^{\bullet-}$, and the first and last bands downshift to 1556 and 1456 cm^{-1}, respectively, upon replacement of Q_A with ^{13}C-Q_{10}. The corresponding three bands have been identified at 1613, 1532, and 1489 cm^{-1} for $Q_B^{\bullet-}$, and the first and last bands downshift to 1555 and 1462 cm^{-1}, respectively, with ^{13}C-Q_{10}. In both cases, the ^{13}C-isotopic shifts of the second band were obscure owing to weakness of bands, but they were evidently shifted with the ^{13}C-$Q_A^{\bullet-}$ and ^{13}C-$Q_B^{\bullet-}$. Figure 1(b) gives the RR spectra in 1300−1760 cm^{-1} region of $Q_{10}^{\bullet-}$ in acetone (A), dichloromethane (B), and ethanol (C). Only three prominent bands were observed around 1610, 1520, and 1490 cm^{-1} for $Q_{10}^{\bullet-}$ in this region. The relative intensities and frequencies of the three bands in organic solvents are similar to those of the RCs, suggesting that the distribution of an extra negative charge of $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ is similar to that of a quinone anion radical in organic solvents.

The bands at 1486 and 1489 cm^{-1} for $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ exhibit downshifts by 30 and 27 cm^{-1}, respectively, for RCs with ^{13}C-Q_{10}. This band is assigned to ν_{7a} (in Wilson's notation), that is, C═O stretch weakly coupled with C═C stretches owing to the agreement of the ^{13}C-isotopic frequency shifts [$\Delta\nu(^{13}C)$] with those calculated for the predominant C═O stretch of $BQ^{\bullet-}$ (1,4-benzoquinone).[13] The $\Delta\nu(^{13}C)$ of the 1610 cm^{-1} band is close to that calculated for ν_{8a} of $BQ^{\bullet-}$, that is, C═C stretch weakly coupled with C═O stretch.[13] The remaining band around 1525 cm^{-1} probably arises from the C═C stretch (ν_{19b}). These assignments are somewhat different from those based on recent FTIR studies[10] and will be addressed elsewhere.[18] An interesting feature to be noted is that $\Delta\nu(^{13}C)$s of ν_{7a} and ν_{8a} exhibit definite differences between $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$, indicating that the coupling between C═C and C═O stretches in these two modes *in vivo* are different and thus that interactions with protein surroundings differ between $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$.

The frequency of ν_{7a} should be sensitive to the strength of hydrogen bonds formed with the C═O groups of $Q_A^{\bullet-}$ or $Q_B^{\bullet-}$. Indeed, for $BQ^{\bullet-}$ and $Q_{10}^{\bullet-}$ in various organic solvents, a clear linear correlation has been found between the ν_{7a} frequencies and the acceptor number (AN) of solvents.[13,18] The ν_{7a}

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S0002-7863(96)03550-0 CCC: $14.00

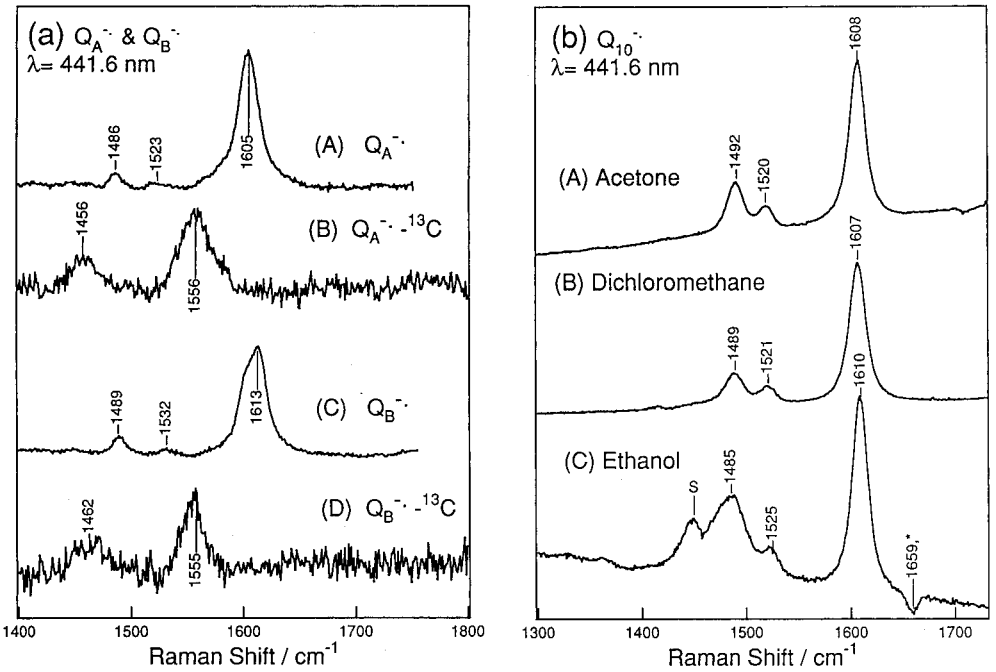

Figure 1. (a) RR spectra of native $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ in *Rb. sphaeroides* RCs and their ^{13}C-isotopomers excited at 441.6 nm, which were obtained by calculating difference spectra between photooxidized and chemically oxidized RCs to cancel the contributions from the special dimer cation. (A) RR spectrum of $Q_A^{\bullet-}$, which was obtained with Q_B-deficient RCs. (B) The RR spectrum of ^{13}C-labeled $Q_A^{\bullet-}$ observed for RCs reconstituted with ^{13}C-labeled Q_{10}. The spectra were obtained with RCs containing Q_B but in the presence of an inhibitor (*o*-phenanthroline, 30 mM) for the electron transfer between Q_A and Q_B. (C) RR spectrum of $Q_B^{\bullet-}$. (D) The RR spectrum of ^{13}C-labeled $Q_B^{\bullet-}$ obtained for RCs reconstituted with ^{13}C-labeled Q_{10}. In all measurements samples were contained in a spinning cell (1800 rpm) at room temperature. Exposure time was 300 s, and a sample was replaced with a fresh one every two measurements. Each spectrum is represented as the average of six measurements. Laser power, 3 mW at sample point. The concentrations of RCs was about 25 μM in 10 mM Tris-HCl, 0.03% LDAO, pH = 8 buffer with 100 mM NaCl. The pump light at 590 nm was generated through an Ar^+ ion laser (Spectra Physics, Stablitie 2017)-pumped dye laser (Spectra Physics, Model 375) with rhodamine 6G. (b) RR spectra of $Q_{10}^{\bullet-}$ in organic solvents upon excitation at 441.6 nm. The solvents bands were subtracted. The bands marked with S and an asterisk arise from a residual solvent band and a laser emission band, respectively. Laser power was 3 mW at sample, and the exposure time was 300 s. Each spectrum is an average of two measurements. The concentrations of Q_{10} before electrolysis were about 0.5 mM.

frequency of $Q_A^{\bullet-}$ is lower than that of $Q_B^{\bullet-}$ by 3 cm^{-1}, which means that the strength of H-bonds between solvent and C=O groups could be somewhat stronger for $Q_A^{\bullet-}$ than for $Q_B^{\bullet-}$. Furthermore, the ν_{7a} of $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ in RCs are higher than that of $Q_{10}^{\bullet-}$ in ethanol by 1 and 4 cm^{-1}, respectively, suggesting that the H-bonds formed with C=O groups of $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ could be slightly weaker in proteins compared with those of $Q_{10}^{\bullet-}$ in ethanol. Similar conclusions concerning the relative strengths of the C=O hydrogen bonds to $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ were pointed out by ENDOR[8] and IR studies.[9]

The RR spectra of the semiquinones greatly enhance the peak near 1610 cm^{-1}, making this a useful probe for the quinone structure. The frequency of this band (ν_{8a}) is higher for $Q_B^{\bullet-}$ than those of $Q_A^{\bullet-}$ by 8 cm^{-1}. The somewhat weaker band near 1530 cm^{-1} (ν_{19b}) also displays a similar shift to higher frequency in $Q_B^{\bullet-}$. In the model systems the C=C stretching frequencies (ν_{8a} and ν_{19b}) of $BQ^{\bullet-}$ and $Q_{10}^{\bullet-}$ exhibit linear upshifts as the increase of solvent AN.[18] This means that the increased electrophilic interactions of solvent raise the C=C sretching frequencies, suggesting the presence of interactions of solvent with π-electrons of quinone rings.[19] Accordingly, one of interpretations for the significant differences in the C=C stretching frequencies between $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ might be that the interactions of π-electrons of quinone with their surroundings are different between $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$.

Two possible differences in the environments of the quinones include the contacts of aromatic residues with $Q_A^{\bullet-}$ and the hydrogen bonds between water and $Q_B^{\bullet-}$. Two aromatic residues, Trp (M252) and Phe (L216), which are in van der Waals contact with π-electrons of Q_A and Q_B, respectively,[6] may yield possible differences in the magnitude of such $\pi-\pi$ interactions and thus in the C=C stretching frequencies between $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ *in vivo*. According to the X-ray structure of stationary RCs,[6] the empty space around the Q_B is filled partly by water. The water molecules may translate and rotate upon the formation of $Q_B^{\bullet-}$. However, it seems also likely to attribute the frequency differences between $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ to the H-bonds formed between π-electrons of $Q_B^{\bullet-}$ and the water molecule-(s). Such putative H-bond(s) between π-electrons of quinones and water molecule(s),[19] if any, could play a key role to stabilize the negative charge of $Q_B^{\bullet-}$ and assure the electron transfer from Q_A to Q_B. In conclusion, the first selective observation of RR spectra of $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$ suggests that the strength of ordinary H-bonds formed by C=O groups of $Q_A^{\bullet-}$ could be somewhat stronger than those by $Q_B^{\bullet-}$ and that the H-bonds in RCs are slightly weaker than those of $Q_{10}^{\bullet-}$ in ethanol.

Acknowledgment. This study was supported by Grant-in-Aids for Scientific Research on Priority Areas (08249106) to T. K. from the Ministry of Education, Science, Culture and Sports Japan and by NIH Grant (GM41637) to M.O. X.Z. was supported by the postdoctoral fellowship of Japan Society for Promotion of Science. We thank Ed Abresch for preparation of RCs and ^{13}C-labeled quinones.

JA963550Z

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