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Light-Induced Charge Separation in *Rhodopseudomonas viridis* Reaction Centers Monitored by Fourier-Transform Infrared Difference Spectroscopy: The Quinone Vibrations[†]

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ABSTRACT: Static FTIR light-induced difference spectra have been recorded for reaction centers from *Rhodopseudomonas viridis* in the following charge-separated states: $P^+Q_A^- - PQ_A$, $P^+Q_B^- - PQ_B$, $I^- - I$, $I^-Q_A^- - IQ_A$, and $I^-Q_A^{2-} - IQ_A$. A comparison of the $I^- - I$ difference spectra with the $I^-Q_A^- - IQ_A$ difference spectra reveals new bands which can be assigned to Q_A^- vibrations; these vibrations are also observed in the $P^+Q_A^- - PQ_A$ and $P^+Q_B^- - PQ_B$ difference spectra. Through an analysis of all of the static difference spectra, the electron-transfer pathway can be monitored in the infrared from the primary donor, P, to the secondary acceptor, Q_B , via the intermediate acceptor, I, and the primary acceptor, Q_A . The difference spectra are dominated by absorbance changes of prosthetic groups, with very few identifiable contributions from amino acids and little overall structural change in the protein backbone, involving only one or two residues for the various charge-separated states. Oxidation of the primary donor in the reaction center shows the characteristic absorbance changes of the 9-keto and 10-ester carbonyl groups observed upon oxidation of bacteriochlorophyll *b* in a non-hydrogen-bonded environment [Ballschmiter, K. H., & Katz, J. J. (1969) *J. Am. Chem. Soc.* 91, 2661-2677]. Reduction of the quinones in the reaction center yields absorbance changes of the carbonyls observed during reduction of quinones in a hydrogen-bonded environment [Bauscher, M., Nabadryk, E., Bagley, K., Breton, J., & Mäntele, W. (1990) *FEBS Lett.* 261, 191-195]. It is thus concluded that the protein acts as an optimized solvent to facilitate electron transfer from the primary donor to the secondary quinone acceptor.

One of the most interesting features of the bacterial photosynthetic reaction center is its efficiency in converting light energy into chemical potential. This energy conversion is accomplished by a series of electron-transfer reactions originating at a noncovalently bound bacteriochlorophyll dimer (P^I) and terminating at a quinone acceptor (Q_B); the quantum yield for photons absorbed by P resulting in electron transfer to Q_B is close to 100% [for reviews, see Feher and Okamura (1978); Hoff (1982), and Feher et al. (1989)]. The reaction center from the purple bacterium *Rhodopseudomonas viridis* contains 4 protein subunits, called L, M, H, and cytochrome, and 14 major cofactors: 4 heme groups covalently attached to the cytochrome subunit; 4 bacteriochlorophylls *b*, 2 bacteriopheophytins *b*, 1 menaquinone 9, 1 ubiquinone 9, 1 non-heme Fe, and 1 carotenoid molecule associated with the subunits L and M. The crystallization (Michel, 1982) of the *Rps. viridis* reaction center and subsequent X-ray diffraction analysis (Deisenhofer et al., 1984, 1985) have presented a detailed picture of the ground (dark) state, represented as PIQ_AQ_B . Absorption of a photon by a special pair populates the lowest electronic state, P^* , which lasts for 2.8 ps (Fleming

et al., 1988). Measurements on *Rhodobacter sphaeroides* reaction centers indicate that the relaxation of this state is concomitant with the transfer of an electron to the accessory bacteriochlorophyll in the L branch (BC_{LA}), forming $P^+BC_{LA}^-$ (Holzapfel et al., 1989). From BC_{LA} , the electron is transferred to the bacteriopheophytin molecule of the L branch (BP_L or I) in 900 fs (Holzapfel et al., 1989). Preliminary measurements on *Rps. viridis* reaction centers imply a time constant of 650 fs for the electron transfer from BC_{LA} to BP_L (Dressler et al., 1990). An alternative model, consisting of a single-step electron transfer from P to BP_L , has also been proposed (Kirmaier & Holten, 1990). From BP_L , an electron is transferred to Q_A in approximately 200 ps [see Woodbury et al. (1985) and references cited therein] and finally to Q_B in 6-30 μ s in *Rps. viridis* (Carithers & Parson, 1975) and in about 100 μ s in *Rb. sphaeroides* (Vermeiglio & Clayton, 1977). Although these charge separation steps are kinetically well characterized, little is known about the role of the protein in the stabilization of the separated charges and whether protein-induced dipoles and/or conformational changes contribute to these processes. Conformational changes have been invoked to explain the recombination kinetics of *Rb. sphaeroides* re-

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¹ Abbreviations: FTIR, Fourier-transform infrared; P, primary donor (bacteriochlorophyll special pair); I, intermediate acceptor (bacteriopheophytin); Q_A , primary quinone acceptor; Q_B , secondary quinone acceptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; BC_{LA} , accessory bacteriochlorophyll in the L branch; BP_L , bacteriopheophytin in the L branch; BP_M , bacteriopheophytin in the M branch; LDAO, *N*-lauryl-*N,N*-dimethylamine *N*-oxide; HPLC, high-pressure liquid chromatography; BChl_b, bacteriochlorophyll *b*; BPh_b, bacteriopheophytin *b*; Q, ubiquinone 0.

action centers frozen during illumination (Kleinfeld et al., 1984), the low-temperature recombination kinetics of *Rb. sphaeroides* and *Rhodospirillum rubrum* reaction centers frozen in the dark (Parot et al., 1987), and the changes in linear dichroism spectra upon charge separation in *Rps. viridis* reaction centers (Vermeglio & Paillotin, 1982). Recently, Robert and Lutz (1988) used resonance Raman spectroscopy to provide specific evidence of light-induced conformational changes in *Rb. sphaeroides* reaction centers occurring upon photooxidation of P. However, protein changes occurring upon quinone reduction have not yet been investigated.

In *Rps. viridis* reaction centers, reduction of either of the two quinone acceptors can be distinguished by a variety of spectroscopic methods: UV-visible spectra of Q_A^- and Q_B^- differ substantially in the near-IR, indicating that Q_A^- induces an electrochromic effect on the bacteriopheophytin in the L subunit while Q_B^- predominantly affects the bacteropheophytin in the M subunit (Shopes & Wraight, 1985). EPR measurements show that both Q_A^- and Q_B^- interact magnetically with the Fe(II) atom but that these interactions are distinctly different for the two quinones (Rutherford & Evans, 1979). Furthermore, the Fe-quinone interactions indicate a role for the Fe(II) atom in electron transfer from Q_A to Q_B , but studies on metal-substituted *Rb. sphaeroides* reaction centers (Debus et al., 1986; Buchanan & Dismukes, 1987) have revealed that the Fe(II) atom plays no electronic role in electron transfer nor does it mediate the redox potential of Q_A (Buchanan et al., 1988). The function of the Fe(II) atom is not yet fully understood nor are specific details known concerning the role of the protein in electron transfer from Q_A to Q_B .

It has been shown that the technique of FTIR difference spectroscopy can reveal changes on the order of a single bond in chromophores, protein side groups, and the protein backbone [for a review, see Braimann and Rothschild (1988)]. Static difference spectra are taken between a ground state and an activated state stabilized through continuous illumination, resulting in difference spectra where all peaks arise from protein and chromophore changes due to photochemical activation. In photosynthetic reaction centers, because the electron-transfer reactions are all several orders of magnitude slower than the forward reactions (Wraight & Clayton, 1974), it is possible to stabilize several of the charge-separated states with continuous illumination. *Rps. viridis* reaction centers in which the cytochrome subunit is oxidized yield $P^+Q_A^-$ or $P^+Q_AQ_B^-$, depending upon preparation conditions, with steady-state illumination (Prince et al., 1977). When reaction centers are reduced with dithionite, all four hemes in the cytochrome subunit and both quinones are reduced, allowing the photochemical trapping of PI^- (Shuvalov & Klimov, 1976; Tropper et al., 1977; Prince et al., 1977). In contrast, ascorbate reduces only the two high-potential hemes (Dracheva et al., 1986); illumination under these conditions initially produces the state $PI^-Q_A^-$ and with continued illumination the state $PI^-Q_A^{2-}$ (van Wijk et al., 1986).

FTIR difference spectroscopy has been used previously to assign changes in the primary donor occurring upon photooxidation in reconstituted reaction centers (Mäntele et al., 1985) and in reaction center crystals (Gerwert et al., 1988). Specifically, H-bond changes for the ester and $C_9=O$ keto groups were identified (Mäntele et al., 1985; Gerwert et al., 1988). A comparison with structural data (Deisenhofer et al., 1984, 1985) has shown that the observed non-hydrogen-bonded C_9 keto carbonyl group can most likely be assigned to the bacteriochlorophyll *b* molecule of the special pair situated in the M subunit (Gerwert et al., 1988). In these experiments,

however, the contributions from reduced quinone molecules were not specifically characterized. Here we report FTIR difference spectra of reconstituted reaction centers for the charge-separated states $P^+Q_A^- - PQ_A$, $P^+Q_B^- - PQ_B$, $I^- - I$, $I^-Q_A^- - IQ_A$, and $I^-Q_A^{2-} - IQ_A$. Through the static difference spectra, the electron-transfer pathway can be followed from P to the secondary electron acceptor, Q_B , via the intermediate acceptor, I, and Q_A . A detailed analysis of the results obtained allows the determination of the roles of the protein and chromophores in charge stabilization.

EXPERIMENTAL PROCEDURES

Reaction centers from *Rps. viridis* (strain DSM 133) were isolated and purified according to Michel (1982), except that the purified reaction centers in 0.1% LDAO (Fluka, Neu-Ulm)/20 mM phosphate buffer, pH 8, were bound to a small anion-exchange column, washed with 0.7% CHAPS (Serva, Heidelberg)/5 mM HEPES (pH 8), and eluted with 0.7% CHAPS/5 mM HEPES/200 mM KCl. Reaction centers were incorporated into soybean phosphatidylcholine vesicles (Cook et al., 1986), with a final lipid concentration of 10 mg/mL and a reaction center concentration of 34 μ M. For samples containing only Q_A , 5 mM tertbutyrn (Riedel-de Haen, Seelze) was routinely added. For samples containing Q_B , ubiquinone 0 (Sigma, Deisenhofen) was dissolved in CHAPS and added in 5 \times excess according to the method of Okamura et al. (1975). This method has been shown to reconstitute 95% Q_B in *Rps. viridis* (Sinning, 1989). Ascorbate reduction was performed by adding 5 mM sodium ascorbate to reaction centers prior to incorporation into lipid vesicles and subsequent dialysis against 5 mM HEPES/5 mM ascorbate. Dithionite reduction was performed by adding 10 μ L of 100 mM HEPES (pH 9)/50 mM $Na_2S_2O_4$ directly to a N_2 -dried lipid film.

Reaction center samples were analyzed for photochemical activity with a Zeiss UMSP 80 microspectrophotometer using actinic illumination $\lambda > 700$ nm. Infrared spectra were taken on a Bruker IFS 88 instrument. A total of 1024 scans were averaged for each spectrum. The home-built sample holder was thermostabilized at 275 K for all measurements. The difference was taken between absorbance spectra recorded in the dark and during steady-state illumination with actinic light > 700 nm. Control experiments using 960-nm excitation provided by an interference filter yielded identical difference spectra. Subtraction of difference spectra was performed as described earlier (Gerwert et al., 1989). This takes into account differing amounts of reacting material in the two measurements and base line drifts between the two difference spectra.

RESULTS

Figure 1 compares the light-induced difference spectra (A) without and (B) with Q_B reconstituted, arising from the transitions $PQ_A \rightarrow P^+Q_A^-$ and $PQ_B \rightarrow P^+Q_B^-$. The high reproducibility of the difference spectra can be seen not only in the large absorbance bands at 1753, 1712, 1552, 1477, 1306, and 1196 cm^{-1} but also in the smaller absorbances between 1412 and 1193 cm^{-1} . Similar difference spectra recorded for reaction center crystals with and without Q_B reconstituted have been reported (Buchanan et al., 1990a) and compare well with the difference spectra for reaction centers reconstituted into lipids vesicles (Figure 1). In the crystal difference spectra, deviations between the two types of difference spectra are seen at 1477/1456 cm^{-1} and could be caused by vibrations of the quinone radical (Tripathie, 1981). The difference spectra for reconstituted reaction centers have an improved signal-to-noise ratio as compared to the crystal difference spectra, thus in-

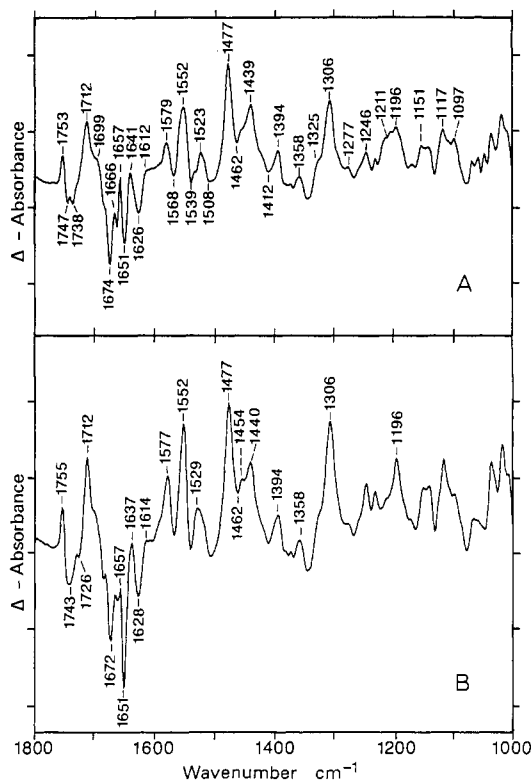


FIGURE 1: FTIR light-induced difference spectra for *Rps. viridis* reaction centers in H_2O . (A) Difference spectrum for reaction centers containing only Q_A^- ; the difference is taken between PQ_A^- and $P^+Q_A^-$ states. (B) Difference spectrum for reaction centers reconstituted with Q_B^- ; the difference is taken between $PQ_AQ_B^-$ and $P^+Q_AQ_B^-$ ($T = 275$ K; resolution = 4 cm^{-1}).

creasing the probability of seeing small changes due to Q_A^- or Q_B^- .

Figure 1A shows a difference spectrum recorded for Q_A^- -containing reaction centers; the difference is taken between the ground state, PQ_A^- , and the charge-separated state stabilized by continuous illumination at 270 K, $P^+Q_A^-$. This difference spectrum roughly agrees with a $P^+Q_A^- - PQ_A^-$ difference spectrum taken at 100 K (Nabedryk et al., 1990); however, some significant deviations are observed: the band at 1720 cm^{-1} observed at 100 K seems to be shifted in Figure 1A to approximately 1738 cm^{-1} , a broader negative band at about 1670 cm^{-1} (Figure 1A) leads to a larger absorbance band at 1674 cm^{-1} , and the positive band at 1630 cm^{-1} observed at 100 K is missing in Figure 1A. Additional bands at 1523 and 1439 cm^{-1} are observed in Figure 1A which do not appear in the 100 K difference spectrum. These discrepancies might be explained by the different temperatures at which the data were recorded.

In Figure 1B, a difference spectrum is shown for Q_B^- -containing reaction centers, arising from the transition $PQ_AQ_B^- \rightarrow P^+Q_AQ_B^-$ (subsequently referred to as a $P^+Q_B^- - PQ_B^-$ difference spectrum). Striking is the great similarity to the $P^+Q_A^- - PQ_A^-$ difference spectrum in Figure 1A. A comparison with a $P^+Q_B^- - PQ_B^-$ difference spectrum recorded under similar conditions (Nabedryk et al., 1990) reveals significant deviations at approximately 1743 , 1672 , 1657 , 1628 , 1552 , 1529 , 1440 , and 1394 cm^{-1} (frequencies refer to Figure 1B). The lack of absorbance bands at 1440 and 1394 cm^{-1} in Nabedryk et al. (1990) is important because they represent quinone vibrations (vide infra).

In Figure 1A, the band shifting from approximately 1740 to 1753 cm^{-1} represents the characteristic absorbance change of a C_{10} carbomethoxy ester carbonyl vibration for bacterio-

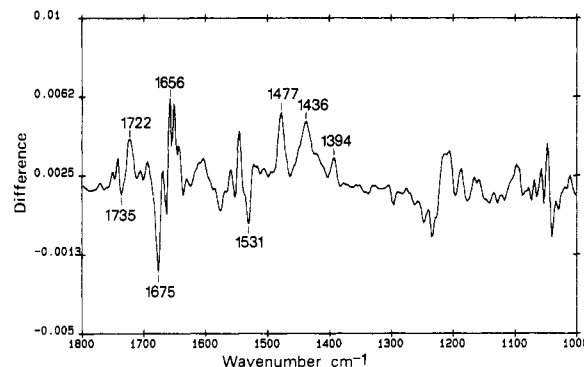


FIGURE 2: Subtraction of $P^+Q_B^- - PQ_B^-$ from $P^+Q_A^- - PQ_A^-$ difference spectra for reaction centers in H_2O . Positive bands are due to $Q_A^- - Q_B^-$, and negative bands are due to $Q_A^- - Q_B^-$.

chlorophyll *b* upon cation formation in a non-hydrogen-bonded environment (Mäntele et al., 1988). The band shifting from 1674 to 1712 cm^{-1} represents the characteristic absorbance change of a C_9 keto carbonyl vibration for bacteriochlorophyll *b* upon cation formation also in a non-hydrogen-bonded environment (Mäntele et al., 1988). The absorbance change at $1674/1712\text{ cm}^{-1}$ is assigned to the 9-keto group of BC_{MP} (special-pair bacteriochlorophyll situated more in the M subunit) because the corresponding group of the L subunit is most likely hydrogen-bonded and would absorb at lower frequencies (Gerwert et al., 1988).

Although some of the absorbance bands observed between 1700 and 1600 cm^{-1} could be caused by vibrations of the primary donor, vibrations from the protein backbone, water, and the quinones would also be expected in this frequency range. Therefore, band assignments in this range are only possible with isotopic labeling or site-directed mutagenesis. The bands at 1552 and 1523 cm^{-1} are also observed in bacteriochlorophyll *b* during cation formation (Mäntele et al., 1988) and are therefore tentatively assigned to primary donor $C=C$ stretching vibrations. In the range from 1500 to 1400 cm^{-1} only very small absorbance changes are detected upon oxidation of bacteriochlorophyll *b*; therefore, absorbance changes in this spectral region are most likely not caused by bacteriochlorophyll vibrations.

A comparison of $P^+Q_A^- - PQ_A^-$ and $P^+Q_B^- - PQ_B^-$ difference spectra should yield agreement for absorbance changes caused by oxidation of the primary donor and differences for absorbance changes caused by reduction of the quinones. A comparison of Q_A^- - and Q_B^- -containing samples was made for difference spectra showing similar intensities for the 1306-cm^{-1} band. The absorbance change at 1306 cm^{-1} is indicative of P^+ formation. FTIR difference spectra of reaction centers containing reduced cytochromes show light-induced absorbance changes due to cytochrome oxidation in the amide I region around 1640 cm^{-1} and in the amide II region around 1550 cm^{-1} , as well as the disappearance of the 1306-cm^{-1} band (Brudler and Gerwert, private communication). The $P^+Q_B^- - PQ_B^-$ difference spectra deviate from the $P^+Q_A^- - PQ_A^-$ difference spectra at 1743 cm^{-1} , at 1726 cm^{-1} , in a broader negative band around 1650 cm^{-1} leading to decreases at 1657 and 1651 cm^{-1} , and at increases in intensity at 1577 , 1477 , 1454 , and 1394 cm^{-1} (Figure 1B). The absorbance change at 1211 cm^{-1} (Figure 1A) is due to strong buffer absorbance and therefore is not specific to Q_A or Q_B vibrations.

In order to visualize the deviations between the $P^+Q_A^- - PQ_A^-$ and $P^+Q_B^- - PQ_B^-$ difference spectra, a subtraction was performed (Figure 2). The absorbance changes caused by oxidation of the primary donor should be canceled, and only

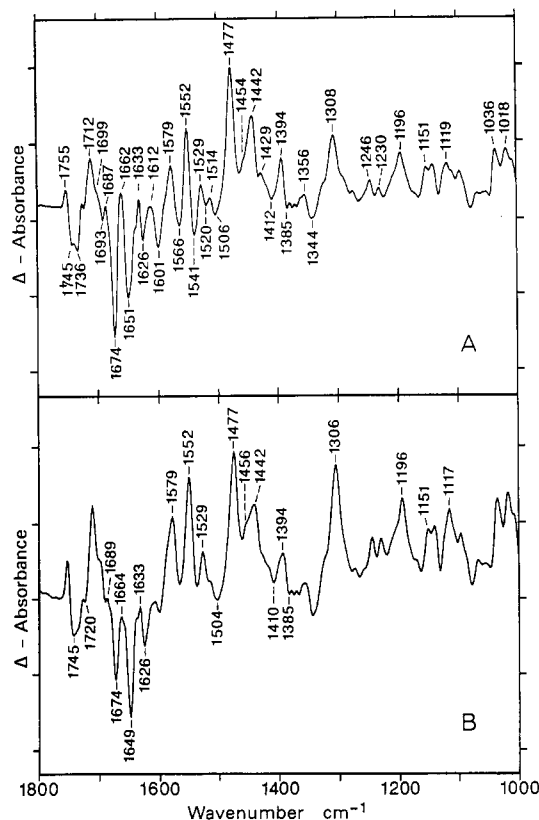


FIGURE 3: Conditions as in Figure 1, except that samples were placed in hydration chambers with $^2\text{H}_2\text{O}$ and equilibrated for 48 h to achieve maximum deuteration. (A) Difference spectrum for Q_A -containing reaction centers. (B) Difference spectrum for Q_B -containing reaction centers.

contributions of the quinones should be present. The subtraction procedure has been described by Gerwert et al. (1989); here positive bands are due to $\text{Q}_\text{A}^- - \text{Q}_\text{B}$, and negative bands are due to $\text{Q}_\text{A} - \text{Q}_\text{B}^-$. Due to the large background absorbance, the amide I and amide II regions must be interpreted with caution. Clear effects are the difference band at 1735/1722 cm^{-1} , the negative band at 1675 cm^{-1} , the band pattern around 1530/1540 cm^{-1} , and the three bands at 1477, 1436, and 1394 cm^{-1} .

To more accurately search for quinone $\text{C}=\text{O}$ contributions in the 1700–1600 cm^{-1} region and to verify the small differences discussed above, $^2\text{H}_2\text{O}$ exchange was performed in hydration chambers for samples containing only Q_A and for samples reconstituted with Q_B . $^2\text{H}_2\text{O}$ does not absorb strongly between 1700 and 1600 cm^{-1} (in contrast to H_2O), and any changes observed in this region would be expected to arise from the protein or chromophoric groups. The lower background absorbance of $^2\text{H}_2\text{O}$ should additionally lead to a better signal-to-noise ratio. Figure 3 shows difference spectra recorded in $^2\text{H}_2\text{O}$ for the $\text{PQ}_\text{A} \rightarrow \text{P}^+\text{Q}_\text{A}^-$ and $\text{PQ}_\text{B} \rightarrow \text{P}^+\text{Q}_\text{B}^-$ transitions. A comparison of the $\text{P}^+\text{Q}_\text{A}^- - \text{PQ}_\text{A}$ difference spectrum taken in $^2\text{H}_2\text{O}$ (Figure 3A) with the difference spectrum from Figure 1A reveals three new positive bands at 1687, 1662, and 1633 cm^{-1} , an additional negative band at 1601 cm^{-1} , and an increase at 1579 cm^{-1} . The band in Figure 1A at 1523 cm^{-1} seems to be upshifted to 1529 cm^{-1} . A comparison of $\text{P}^+\text{Q}_\text{B}^- - \text{PQ}_\text{B}$ difference spectra taken in H_2O and $^2\text{H}_2\text{O}$ shows the same deviations at 1689, 1664, 1633, 1601, and 1579 cm^{-1} , as already seen in the $\text{P}^+\text{Q}_\text{A}^- - \text{PQ}_\text{A}$ difference spectra.

The reduced background absorption of $^2\text{H}_2\text{O}$ between 1700 and 1600 cm^{-1} reveals deviations between Q_A - and Q_B -containing samples (Figure 3) at 1736, 1674, 1651, 1541, 1477, 1454, and 1394 cm^{-1} . A subtraction of $\text{P}^+\text{Q}_\text{A}^- - \text{PQ}_\text{A}$ and

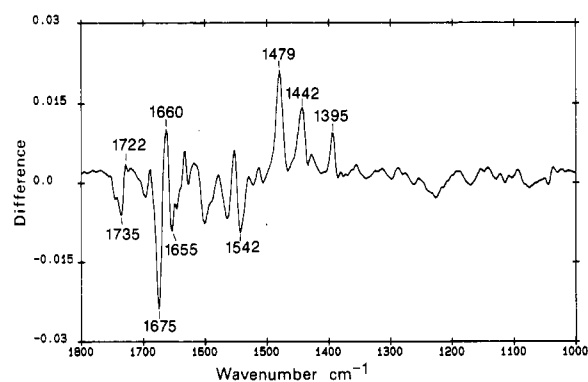


FIGURE 4: Subtraction of $\text{P}^+\text{Q}_\text{B}^- - \text{PQ}_\text{B}$ from $\text{P}^+\text{Q}_\text{A}^- - \text{PQ}_\text{A}$ difference spectra for reaction centers in $^2\text{H}_2\text{O}$. Positive bands are due to $\text{Q}_\text{A}^- - \text{Q}_\text{B}$, and negative bands are due to $\text{Q}_\text{A} - \text{Q}_\text{B}^-$.

$\text{P}^+\text{Q}_\text{B}^- - \text{PQ}_\text{B}$ difference spectra in $^2\text{H}_2\text{O}$ was performed (Figure 4). The error of the subtraction can be estimated by the noise seen below 1395 cm^{-1} . Figure 4 shows differences between Q_A^- and Q_B^- in the carbonyl region at 1735 and 1722 cm^{-1} , and in an intense negative band at 1675 cm^{-1} . Again a difference band around 1540 cm^{-1} and the three positive bands at 1479, 1442, and 1395 cm^{-1} are observed. The absorbance changes caused by quinone reduction, especially the bands at 1735, 1722, 1675, 1479, 1442, and 1395 cm^{-1} , agree quite well in H_2O (Figure 2) and $^2\text{H}_2\text{O}$ (Figure 4).

A second approach to identify absorptions belonging to Q_A^- involves the selective reduction of the high-potential cytochromes (of the tightly bound cytochrome subunit) with ascorbate. In ascorbate-reduced samples containing only Q_A , an electron will be transferred to Q_A in approximately 200 ps (Woodbury et al., 1985). Before this charge-separated state can recombine (time constant of about 1 ms at pH 8; Shopes & Wraight, 1985), the highest potential cytochrome reduces P^+ in 0.32 μs (Dracheva et al., 1988), allowing a second electron to be transferred to I (about 3 ps; Fleming et al., 1988). Thus, steady-state illumination of ascorbate-reduced reaction centers initially produces $\text{PI}^-\text{Q}_\text{A}^-$ (van Wijk et al., 1986), which can be compared to dithionite-reduced samples showing light-induced absorptions arising from PI^- (Trosper et al., 1977; Prince et al., 1977). Continued illumination of dithionite- or ascorbate-reduced samples eventually produces the semiquinone dianion, $\text{PI}^-\text{Q}_\text{A}^{2-}$; this process has been estimated to occur in 95% of the reaction center population in *Rps. viridis* (van Wijk et al., 1986). Steady-state illumination at room temperature of dithionite-reduced reaction centers has also been shown to reduce the bacteriopheophytin in the M subunit (Thorner et al., 1981; Schenck et al., 1981). Although the extent of bacteriopheophytin reduction through continuous illumination of ascorbate-reduced reaction centers is not known, it is likely that BP_M vibrations will contribute to the difference spectra of both dithionite- and ascorbate-reduced reaction center samples. Nevertheless, a subtraction of the dithionite-reduced reaction center difference spectrum from the difference spectrum obtained from ascorbate-reduced reaction centers should cancel bacteriopheophytin absorbance changes and emphasize vibrations arising from $\text{Q}_\text{A}^-/\text{Q}_\text{A}$.

Figure 5 shows the UV-visible light-induced difference spectra for reaction centers in which the cytochrome subunit is oxidized (Figure 5, top panel) and for reaction centers with the high-potential hemes reduced by ascorbate (Figure 5, bottom panel). Both spectra were obtained by continuous illumination with $\lambda > 700$ nm, in a manner analogous to the FTIR difference spectra. In the oxidized preparation (Figure 5, top), illumination stabilizes the charge-separated state P^+Q^- ,

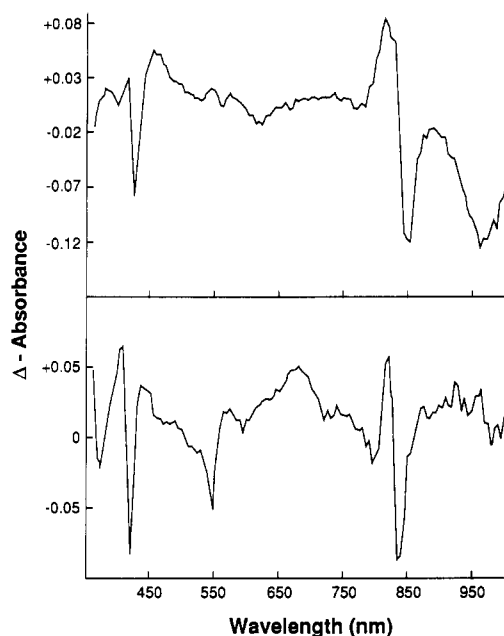


FIGURE 5: UV-visible light-induced difference spectra for *Rps. viridis* reaction centers. (Top) Difference spectrum obtained for untreated Q_A -containing reaction centers; the difference is taken between PQ_A and $P^+Q_A^-$. (Bottom) Difference spectrum for Q_A -containing reaction centers reduced with ascorbate; the difference is taken between PIQ_A^- and $PI^-Q_A^-$ ($T = 295$ K; resolution = 3 nm).

as indicated by a complete bleaching of the 960-nm absorbance band as well as a bleaching of the Q_x (BChl_B) absorbance at 610 nm. The band with a negative lobe at about 850 nm and a positive lobe at 810 nm is thought to be due to a Stark shift of the accessory bacteriochlorophyll monomers and is typical for a P^+Q^- difference spectrum [see, e.g., Knapp et al. (1985)]. In contrast, continuous illumination of the ascorbate-reduced sample yields a bleaching of the BPh_B bands at 545 and 790 nm as well as a large blue shift of the 830-nm band; these changes are indicative of the photochemical trapping of I^- (Trosper et al., 1977; Prince et al., 1977). The shoulder at 530 nm indicates partial reduction also of the bacteriopheophytin in the M subunit; the extent of BP_M reduction as estimated from the absorbance intensity at 530 nm as compared to the intensity at 545 nm is approximately 25%. Since the quinones are not chemically reduced by ascorbate treatment (van Wijk et al., 1986), the appearance of an I^- spectrum upon illumination indicates that Q_A must also be photochemically reduced, resulting in the state $PI^-Q_A^-$. Light-induced difference spectra for dithionite-reduced reaction centers also yield a bleaching of the BPh_B bands at 545 and 790 nm and a blue shift of the 830-nm absorbance, indicating the photochemical trapping of I^- (Buchanan, 1990).

Figure 6A shows a difference spectrum for dithionite-reduced reaction centers arising from the transition $PIQ_A^- \rightarrow PI^-Q_A^-$ (the difference spectrum represents changes caused by the reduction of the intermediate acceptor and is subsequently referred to as $I^- - I$). The carbonyl region is dominated by four negative absorbances at 1745, 1732, 1682, and 1657 cm^{-1} as well as a broad positive absorbance at 1714 cm^{-1} . Other peaks characteristic for the $I^- - I$ difference spectrum are an intense positive absorption at 1597 cm^{-1} as well as smaller positive absorptions at 1552, 1466, and 1371 cm^{-1} . Resonance Raman spectra of BPh_B in methanol show absorptions at 1665, 1601, and 1575 cm^{-1} (Robert et al., 1987), while FTIR difference spectra for electrochemically generated $BPh_B^- - BPh_B$ show positive absorbances at 1658, 1627, 1565, 1483, 1468, 1372, 1345, and 1332 cm^{-1} as well as negative

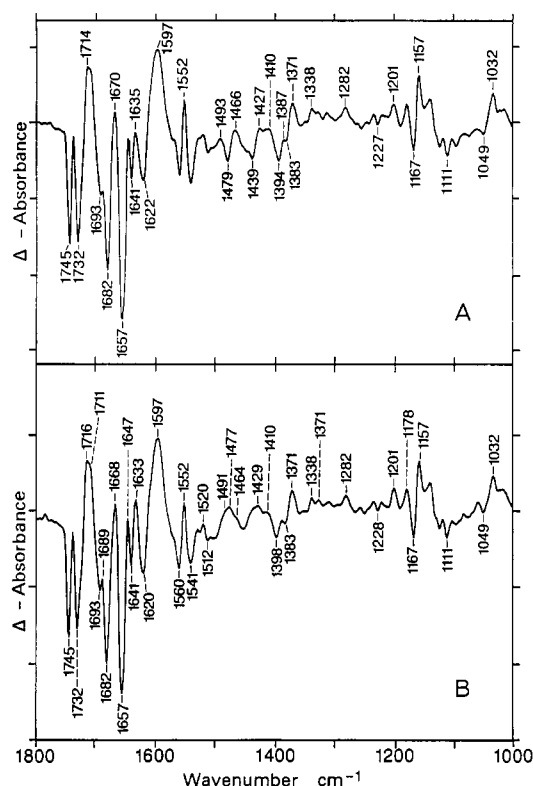


FIGURE 6: FTIR light-induced difference spectra for chemically reduced Q_A -containing reaction centers. (A) Difference spectrum obtained for dithionite-reduced reaction centers; the difference is taken between PIQ_A^- and $PI^-Q_A^-$. (B) Difference spectrum obtained for dithionite-reduced reaction centers illuminated for 5 min prior to recording the "light" spectrum; the difference is taken between PIQ_A^- and $PI^-Q_A^{2-}$ ($T = 275$ K; resolution = 4 cm^{-1}).

absorptions at 1743, 1703, and 1401 cm^{-1} (Mäntele et al., 1988).

The difference spectrum in Figure 6A is similar to an $I^- - I$ difference spectrum published by Mäntele et al. (1988); however, some significant deviations are observed. In Figure 6A, the negative absorbance bands at 1682 and 1657 cm^{-1} have much stronger intensities than the negative bands at 1745 and 1732 cm^{-1} . Additional positive bands are detected at 1493 and 1410 cm^{-1} . Because the difference spectrum in Figure 6A agrees very well below 1300 cm^{-1} with that of Mäntele et al. (1988) and since the $I^- - I$ difference spectra are highly reproducible even for the smaller absorbance changes, the discrepancies seem not to be caused by experimental uncertainties.

Continued illumination of the dithionite-treated reaction centers results in the accumulation of the quinone dianion (Prince et al., 1977) and in reduction of the bacteriopheophytin in the M subunit (Thorner et al., 1981). The corresponding difference spectrum, arising from the transition $PIQ_A^- \rightarrow PI^-Q_A^{2-}$ and also containing contributions from BP_M (subsequently referred to as $I^-Q_A^{2-} - IQ_A^-$), is shown in Figure 6B. The $I^- - I$ and $I^-Q_A^{2-} - IQ_A^-$ difference spectra are in complete agreement except between 1500 and 1400 cm^{-1} ; in this region, positive bands arise at 1477 and 1429 cm^{-1} which likely are due to Q_A^{2-} vibrations. This indicates that the $I^-Q_A^{2-} - IQ_A^-$ difference spectrum is largely dominated by absorbance changes due to reduction of the intermediate electron acceptor. The difference spectra in Figure 6 indicate that in Mäntele et al. (1988) a mixture of an $I^- - I$ and $I^-Q_A^{2-} - IQ_A^-$ is obtained and explains the discrepancies cited above.

The model compound spectra of BPh_B (Mäntele et al., 1988) show the same absorbance changes between 1420 and 1330

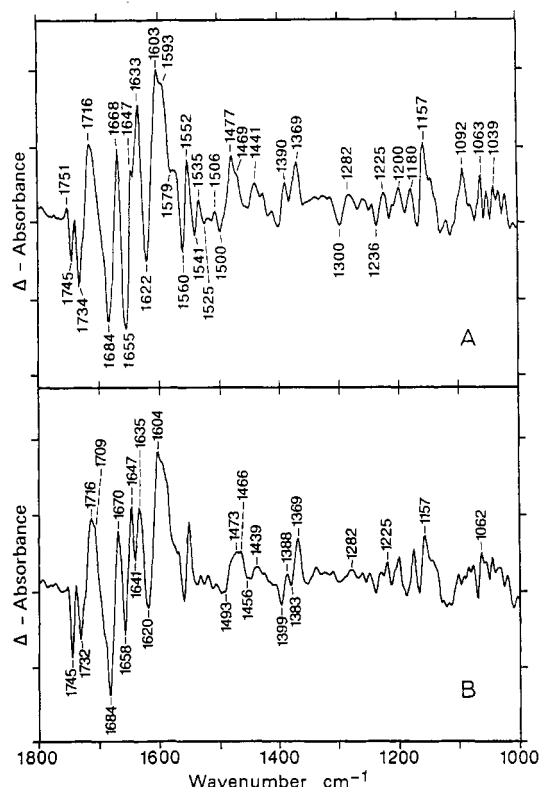


FIGURE 7: FTIR light-induced difference spectra for chemically reduced Q_A -containing reaction centers. (A) Difference spectrum obtained for ascorbate-reduced reaction centers; the difference is taken between PIQ_A and PIQ_A^- . (B) Difference spectrum obtained for ascorbate-reduced reaction centers illuminated for 5 min prior to recording the "light" spectrum; the difference is taken between PIQ_A and PIQ_A^{2-} ($T = 275$ K; resolution = 4 cm^{-1}).

cm^{-1} upon anion formation in an aprotic solvent as are observed in the $I^- - I$ difference spectra. These changes are therefore most likely caused by reduction of the intermediate electron acceptor. Upon anion formation of BPh_B in an aprotic solvent, negative carbonyl bands are observed at 1703 and 1743 cm^{-1} and a positive band at 1727 cm^{-1} . This deviates from the band pattern observed upon reduction of the intermediate electron acceptor; the $I^- - I$ difference spectra reveal two different carbonyl vibrations at 1745 and 1732 cm^{-1} . In contrast to the $P^+Q^- - PQ$ difference spectra, no upshifted ester absorbance band is observed in the BPh_B state for either the model compound difference spectra or the $I^- - I$ reaction center difference spectra. The absorbance band at 1714 cm^{-1} , which is downshifted compared to model compounds, either could represent a downshifted ester carbonyl vibration (indicating hydrogen bonding of this group) or, as is found for the oxidation of the primary donor, could represent a 9-keto carbonyl vibration upshifted from 1682 cm^{-1} , indicating a hydrophobic environment for the keto group. The absorbance changes below 1420 cm^{-1} are not perturbed by pigment-protein interactions in the reaction center complex since the same absorbance changes are observed during BPh_B reduction in aprotic solvents. However, band patterns are observed in the carbonyl region of the reaction center $I^- - I$ difference spectra which deviate from model compound $BPh_B^- - BPh_B$ difference spectra in an aprotic solvent, indicating more extensive interaction with the protein environment.

Figure 7A shows an FTIR difference spectrum for ascorbate-reduced reaction centers, arising from the transition $PIQ_A \rightarrow PIQ_A^-$ (subsequently referred to as an $I^-Q_A^- - IQ_A$ difference spectrum). The difference spectrum is very similar to the $I^- - I$ difference spectrum (Figure 6A); the characteristic

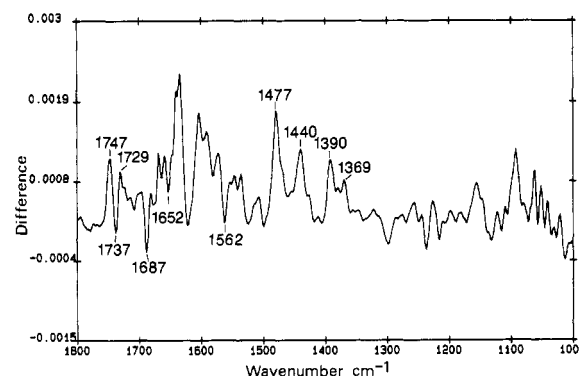


FIGURE 8: Subtraction of $I^- - I$ from $I^-Q_A^- - IQ_A$ difference spectra. Positive bands are due to Q_A^- , and negative bands are due to Q_A .

pattern of four negative peaks at 1745, 1734, 1684, and 1655 cm^{-1} and one broad positive peak at 1716 cm^{-1} indicates the photochemical trapping of I^- . A comparison of the difference spectra in Figures 6A and 7A reveals the same absorbance changes between 1800 and 1500 cm^{-1} except for the increased intensity at 1633 cm^{-1} for the ascorbate-reduced sample (Figure 7A); this intensity change could be caused by phase errors leading to shifts in intensity. Significant deviations are observed between 1500 and 1300 cm^{-1} . In Figure 7A, additional negative bands are seen at 1535 and 1500 cm^{-1} . Larger positive absorbance bands are also observed at 1477, 1441, and 1390 cm^{-1} . Because the only new species stabilized by continuous illumination with ascorbate reduction as compared to dithionite reduction is Q_A^- , the additional positive absorbances in Figure 7A must represent absorbance changes arising from the semiquinone anion radical or affected amino acid residues.

A subtraction of the $I^- - I$ difference spectrum from the $I^-Q_A^- - IQ_A$ difference spectrum was performed to more clearly visualize vibrations due to Q_A^-/Q_A ; this difference spectrum is shown in Figure 8. The similarity of Figure 8 to the subtractions of difference spectra in Figures 2 and 4 is remarkable: absorbance changes in the carbonyl region, now revealed by subtraction of the larger bacteriopheophytin absorbances in this region, are seen at 1747, 1737/1729, 1687, and 1652 cm^{-1} . A negative absorbance at 1562 cm^{-1} is apparent, and the absorbance changes at 1477, 1440, and 1390 cm^{-1} are distinctly revealed through the subtraction and agree with Figures 2 and 4. This is surprising because in Figures 2 and 4 absorbance changes caused by reduction of Q_B should also be present, yet no clear deviations can be identified.

Although the infrared spectra of quinones have not been extensively studied, some data exist for neutral ubiquinones and menaquinones (Pennock, 1965) which are useful for the analysis of reaction center difference spectra. Neutral ubiquinones and menaquinones generally show an intense band at approximately 1650 cm^{-1} which is attributed to the quinone $C=O$ stretch. The $C=C$ stretch of the quinone ring occurs at about 1610 cm^{-1} , and aromatic vibrations occur at about 1590 cm^{-1} . Additionally, menaquinones show absorbance frequencies characteristic of the naphthoquinone nucleus at 1667, 1587, 1299, and 714 cm^{-1} . Ubiquinones show bands at 1259 and 1094 cm^{-1} which have been suggested to arise from the two methoxy groups in the quinone ring (Pennock, 1965). Spectroelectrochemical model compound studies of ubiquinone 0 (Bauscher et al., 1990) show upon anion formation the disappearance of two bands at 1660 and 1606 cm^{-1} , independent of solvent, and the appearance of a larger band at 1500 cm^{-1} and a smaller band at 1473 cm^{-1} in an aprotic solvent, or a larger band at 1476 cm^{-1} with shoulders at 1438 and 1394 cm^{-1} in a protic solvent. In contrast, BPh_B model

compound studies (Mäntele et al., 1988) show upon anion formation almost no absorbance changes between 1500 and 1400 cm^{-1} with the exception of a small band at 1419 cm^{-1} . Therefore, the additional positive bands observed at 1477, 1441, and 1390 cm^{-1} in the $\text{I-Q}_\text{A}^- - \text{I-Q}_\text{A}$ difference spectrum can most likely be assigned to $\text{C}=\text{O}^-$ vibrations of the semiquinone anion radical due to the good agreement in frequency and intensity with quinone absorbance changes observed in a protic solvent upon anion formation. A comparison of the $\text{I}^- - \text{I}$ (Figure 6A) and $\text{I-Q}_\text{A}^- - \text{I-Q}_\text{A}$ (Figure 7A) difference spectra reveals some dissimilarities in the 1700–1600- cm^{-1} region, but specific assignments require isotopic labeling.

Extended illumination of the ascorbate-reduced sample results in the difference spectrum depicted in Figure 7B. A comparison with Figure 7A shows intensity decreases at 1473, 1466, 1439, and 1410 cm^{-1} . The same spectral features are observed during the formation of Q^{2-} in a protic solvent (Bauscher et al., 1990), indicating the accumulation of Q_A^{2-} in the ascorbate-reduced reaction center sample. Contributions from BP_M^- are probably also present (Thornber et al., 1981). Model compound spectra for Q^{2-} in an aprotic solvent show different spectral features which do not correspond to those observed in reaction center samples. The agreement of the spectral features in Figure 7B with model compound studies for Q^{2-} formation in a protic solvent supports the assignment of the quinone vibrations and the conclusion that Q_A^- is hydrogen-bonded to its environment.

DISCUSSION

FTIR light-induced difference spectra have been recorded in *Rps. viridis* for the following charge-separated states: $\text{P}^+\text{Q}_\text{A}^- - \text{PQ}_\text{A}$, $\text{P}^+\text{Q}_\text{B}^- - \text{PQ}_\text{B}$, $\text{I}^- - \text{I}$, $\text{I-Q}_\text{A}^- - \text{I-Q}_\text{A}$, and $\text{I-Q}_\text{A}^{2-} - \text{I-Q}_\text{A}$. The $\text{P}^+\text{Q}^- - \text{PQ}$ difference spectra are dominated by chromophore absorbances. Spectroelectrochemical studies of bacteriochlorophyll model compounds indicate that only the keto and ester carbonyl vibrations have differing absorbances when oxidation takes place in a hydrogen-bonded as compared to a non-hydrogen-bonded environment. The absorbance changes in the P^+Q^- charge-separated state show the characteristic absorbance changes of the ester and keto carbonyl vibrations of the primary donor in a non-hydrogen-bonded environment.

A lack of specific protein absorbance changes could have two explanations: (1) if the extinction coefficients are lower for reactive side groups than for reactive chromophores, the protein absorptions could be masked by chromophore bands or be too small to be resolved; or (2) the protein does not participate extensively in this charge separation. It has been shown (Gerwert et al., 1989) that extinction coefficients for participating protein groups, in this case an aspartate carbonyl, are of the same order of magnitude as chromophore extinction coefficients. Therefore, it is reasonable to expect that similar protein interactions in the reaction center should be observable if they are significant. Furthermore, the protein backbone undergoes a conformational change involving only one or two residues upon P^+Q^- stabilization, as estimated by the absorbance changes of the amide I and II bands (1647, 1545 cm^{-1}) during photooxidation.

A comparison of the $\text{I}^- - \text{I}$ reaction center difference spectra with $\text{BPh}_\text{B}^- - \text{BPh}_\text{B}$ difference spectra generated electrochemically (Mäntele et al., 1988) reveals the same band patterns below 1420 cm^{-1} for reaction center difference spectra as for $\text{BPh}_\text{B}^- - \text{BPh}_\text{B}$ recorded in an aprotic solvent. Above 1420 cm^{-1} , however, notable deviations between the two types of difference spectra are observed. Electron transfer to BP_M has

been measured in *Rps. viridis* under conditions where BP_L is prerduced; an electron-transfer branching ratio of approximately 200 was measured for k_L/k_M (Tiede et al., 1990). At room temperature with continuous illumination, the accumulation of BP_M^- cannot be excluded (Thornber et al., 1981) but should contribute no more than 25% to the BP_L^- vibrations. The $\text{I}^- - \text{I}$ difference spectra reveal two different carbonyl vibrations at 1745 and 1732 cm^{-1} , as compared to the single carbonyl vibration at 1743 cm^{-1} seen in the model compound spectra. Two explanations could be that (1) the absorbance at 1745 cm^{-1} represents a protonated carboxylic acid (most likely Glu-L104; Nabedryk et al., 1988; Michel et al., 1986) and the absorbance at 1732 cm^{-1} arises from a hydrogen-bonded ester carbonyl group or (2) the bands at 1745 and 1732 cm^{-1} represent non-hydrogen-bonded and hydrogen-bonded ester carbonyl groups, respectively. The former explanation is strengthened by near-IR resonance Raman spectra (Bocian et al., 1987) which also provide evidence of Glu-L104 interaction with the C_9 keto group of BP_L ; a strong band at 1726 cm^{-1} was attributed to a C_{10} carbomethoxy carbonyl group which most likely becomes Raman-active by induced partial double bond character in the C_9C_{10} bond caused by a partial charge redistribution of the $\text{C}_9=\text{O}$ keto group. However, an absorbance band of a protonated carboxylic acid should shift in $^2\text{H}_2\text{O}$ by 10 cm^{-1} as shown in bacteriorhodopsin (Engelhard et al., 1985); such a pronounced shift was not observed by Nabedryk et al. (1990), making the assignment ambiguous. Once again, a small conformational change involving one or two residues takes place upon I^- charge stabilization as monitored by the intensities of the amide I and II bands.

The $\text{I-Q}_\text{A}^- - \text{I-Q}_\text{A}$ reaction center difference spectra compare well with the $\text{I}^- - \text{I}$ difference spectra above 1500 cm^{-1} , indicating that most bands in this spectral region arise from the bacteriopheophytin vibrations. The intensities of the amide I and II bands again indicate conformational changes involving one or two residues upon charge stabilization. It is possible to identify $\text{C}=\text{O}^-$ vibrations of Q_A at 1477, 1440, and 1390 cm^{-1} by comparison with $\text{I}^- - \text{I}$ difference spectra. The identified $\text{C}=\text{O}^-$ vibrations of Q_A^- indicate clearly that the carbonyls of Q_A^- are hydrogen-bonded to their environments in the charge-separated state. No distinguishable carbonyl vibrations for the neutral Q_A or for $\text{Q}_\text{B}/\text{Q}_\text{B}^-$ can be identified. It is possible that Q_B^- vibrations occur at about the same positions as the Q_A^- vibrations or that the Q_B^- vibrations are broadened by more extensive hydrogen bonding to the protein matrix and are masked by Q_A^- vibrations.

Because few specific protein vibrations are seen in all of the difference spectra, it appears that many protein residues must make small contributions to charge solvation rather than large contributions from a few select protein residues. No large conformational changes are observed in any of the charge-separated states stabilized by continuous illumination, indicating that the protein matrix surrounding the chromophores remains rigid throughout electron transfer from P to Q_B . It can be excluded that larger structural rearrangements contribute to the fast forward electron-transfer rates [for a review, see Friesner and Won (1989)]. This conclusion is confirmed by X-ray diffraction experiments comparing dark and illuminated *Rps. viridis* crystals containing Q_B , where no significant differences were seen between data sets collected to 3-Å resolution (Buchanan et al., 1990b).

A molecular dynamics simulation of electron transfer from P to I implies similar conclusions: the P^+I^- charge separation appears to be stabilized by many small, rapid adjustments in the protein, largely accomplished by electrostatic interactions

between partial charges localized on the chromophores and the surrounding protein matrix (Treutlein et al., 1991; Nonnella & Schulten, 1990). This would explain the relatively small protein contributions to the FTIR difference spectra; many small contributions from different types of amino acids would yield smaller absorptions than those found for the chromophores.

The difference spectra of the various charge-separated states suggest that the protein matrix of the reaction center behaves like an optimized solvent, largely hydrophobic in nature surrounding the special pair, still hydrophobic but with some polar protein-pigment interactions around the intermediate acceptor, and hydrophilic in the regions of Q_A and Q_B . The good solvation of Q_A^- and Q_B^- can explain the relatively long lifetimes of the Q_A^- and Q_B^- charge-separated states.

Interestingly, similar results to those presented here for *Rps. viridis* were recently reported for *Rb. sphaeroides* using a different experimental approach (Thibodeau et al., 1990). In these studies, the quinone vibrations in *Rb. sphaeroides* were assigned using time-resolved FTIR spectroscopy; the different recombination times of $P^+Q_A^-$ and $P^+Q_B^-$ allow selection of the quinone vibrations by subtraction at different time intervals. In agreement with our findings in the *Rps. viridis* difference spectra, correspondingly small quinone absorbance changes are observed for *Rb. sphaeroides* as compared to the absorbance changes of the primary donor (P) during charge separation. The similarities between the FTIR studies on *Rps. viridis* and *Rb. sphaeroides* reaction centers indicate very similar intermolecular reactions occurring in both types of reaction centers during charge separation.

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A ^{31}P NMR Study of Mitochondrial Inorganic Phosphate Visibility: Effects of Ca^{2+} , Mn^{2+} , and the pH Gradient[†]

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ABSTRACT: The effects of external pH, temperature, and Ca^{2+} and Mn^{2+} concentrations on the compartmentation and NMR visibility of inorganic phosphate (P_i) were studied in isolated rat liver mitochondria respiring on succinate and glutamate. Mitochondrial matrix P_i is totally visible by NMR at 8 °C and at low external concentrations of P_i . However, when the external P_i concentration is increased above 7 mM, the pH gradient decreases, the amount of matrix P_i increases, and the fraction not observed by NMR increases. Raising the temperature to 25 °C also decreases the pH gradient and the P_i fraction observed by NMR. At physiologically relevant concentrations, Ca^{2+} and Mn^{2+} do not seem to play a major role in matrix P_i NMR invisibility. For Ca^{2+} concentrations above 30 nmol/mg of protein, formation of insoluble complexes will cause loss of P_i signal intensity. For Mn^{2+} concentrations above 2 nmol/mg of protein, the P_i peak can be broadened sufficiently to preclude detection of a high-resolution signal. The results indicate that mitochondrial matrix P_i should be mostly observable up to 25 °C by high-resolution NMR. While the exact nature of the NMR-invisible phosphate in perfused or in vivo liver is yet to be determined, better success at detecting and resolving both P_i pools by NMR is indicated at high field, low temperature, and optimized pulsing conditions.

The phosphorylation potential $[\text{ATP}]/[\text{ADP}][\text{P}_i]$ is frequently used as a measure of the available free energy of the

cellular adenine nucleotide pool and thus is an important means of evaluating cell metabolism. In various tissues, a considerably higher phosphorylation potential is calculated using phosphorus-31 nuclear magnetic resonance (^{31}P NMR)¹ measurements (Iles et al., 1985; Cunningham et al., 1986; Desmoulin et al., 1987) than by using biochemical data (Siess et al., 1982; Soboll et al., 1978; Aw et al., 1987; Klingenberg

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¹ Abbreviations: ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; T_1 , spin-lattice relaxation time; T_2 , spin-spin relaxation time; T_2^* , apparent spin-spin relaxation time including field inhomogeneity contribution; CDTA, *trans*-1,2-diaminocyclohexane-*N,N',N''*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinedithanesulfonic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DMO, 5,5'-dimethylloxazolidine-2,4-dione.