Binding Sites of Quinones in Photosynthetic Bacterial Reaction Centers Investigated by Light-Induced FTIR Difference Spectroscopy: Symmetry of the Carbonyl Interactions and Close Equivalence of the Q_B Vibrations in *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* Probed by Isotope Labeling

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ABSTRACT: The photoreduction of the secondary quinone acceptor Q_B in reaction centers (RCs) of the photosynthetic bacteria *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* has been investigated by light-induced FTIR difference spectroscopy of RCs reconstituted with several isotopically labeled ubiquinones. The labels used were ^{18}O on both carbonyls and ^{13}C either uniformly or selectively at the 1- or the 4-position, i.e., on either one of the two carbonyls. The Q_B^-/Q_B spectra of RCs reconstituted with the isotopically labeled and unlabeled quinones as well as the double differences calculated from these spectra exhibit distinct isotopic shifts for a number of bands attributed to vibrations of Q_B and Q_B^-. The vibrational modes of the quinone in the Q_B site are compared to those of ubiquinone *in vitro*, leading to band assignments for the C═O and C═C vibrations of the neutral Q_B and for the C•••O and C•••C of the semiquinone. The C═O frequency of each of the carbonyls of the unlabeled quinone is revealed at 1641 cm^{-1} for both species. This demonstrates symmetrical and weak hydrogen bonding of the two C═O groups to the protein at the Q_B site. In contrast, the C═C vibrations are not equivalent for selective labeling at C_1 or at C_4, although they both contribute to the ~1617-cm^{-1} band in the Q_B^-/Q_B spectra of the two species. Compared to the vibrations of isolated ubiquinone, the C═C mode of Q_B does not involve displacement of the C_4 carbon atom, while the motion of C_1 is not hindered. Further analysis of the spectra suggests that the protein at the binding site imposes a specific constraint on the methoxy and/or the methyl group proximal to the C_4 carbonyl. The FTIR observations provide compelling evidence for almost identical conformation and identical interactions of the ubiquinone in the Q_B binding site of *Rb. sphaeroides* and *Rp. viridis* in contrast to the X-ray structures, which yield different descriptions for the hydrogen-bonding pattern of Q_B binding. In the semiquinone state, the bonding interactions of the C•••O groups are also symmetrical and the C•••C are inequivalent at C_1 and C_4. However, the interactions are almost the same in the RCs of both species.

In the reaction center (RC)[1] of photosynthetic purple bacteria, two quinone molecules (Q_A, Q_B) play an essential role in coupling the electron and proton transfer reactions leading to the conversion of light energy into chemical energy [for a review, see Feher et al. (1989)]. Following absorption of a photon, electron transfer occurs between a dimer of bacteriochlorophyll molecules and a bacteriopheophytin in ~3 ps. This is followed by electron transfer to Q_A in ~200 ps and then to Q_B in 20−100 μs. Q_A acts as a one-electron acceptor only, while Q_B plays the role of a two-electron gate and can accept two protons before leaving the RC site as a dihydroquinone (QH_2). The empty Q_B site is regenerated by an oxidized ubiquinone from a pool while QH_2 binds to the cytochrome bc_1 complex, where its reoxidation is coupled to the generation of a transmembrane proton gradient.

The crystal structure of the RC of the two species of photosynthetic bacteria that have been investigated the most, namely, *Rhodobacter* (*Rb.*) *sphaeroides* and *Rhodopseudomonas* (*Rp.*) *viridis*, has been solved to different levels of atomic resolution in several laboratories (Michel et al., 1986; Deisenhofer & Michel, 1989; Deisenhofer et al., 1995; Lancaster et al., 1995; Allen et al., 1988; El-Kabbani et al., 1991; Chirino et al., 1994; Ermler et al., 1994). For these two highly homologous proteins, the derived structural models reveal that Q_A and Q_B, which in *Rp. viridis* are menaquinone-9 and ubiquinone-9 (Q_9), respectively, while in *Rb. sphaeroides* they are both Q_{10}, differ significantly in the polarity of their protein binding sites. Notably, the lining of the Q_A site is mostly apolar, while Q_B is surrounded by several protonatable residues. The bonding interactions of Q_A with the protein are relatively well defined (within the ±0.25−0.5-Å precision on the position of the nonhydrogen atoms in the X-ray data) although the details vary among the various structures. In contrast, the description of the binding site of Q_B differs considerably for the various structural models. This variability in the position of Q_B may

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[1] Abbreviations: RC, reaction center; Q_A (Q_B), primary (secondary) quinone acceptor; P, primary electron donor; *Rb., Rhodobacter*; *Rp., Rhodopseudomonas*; FTIR, Fourier transform infrared; Q_n, 2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benzoquinone; DQ (duroquinone), 2,3,5,6-tetramethyl-1,4-benzoquinone; DAD (diaminodurene), 2,3,5,6,-tetramethyl-*p*-phenylenediamine.

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reflect the function of this acceptor which, upon double reduction, leaves the protein so that it must be located near a conduct to the exterior. In a recent study, the X-ray structures of the different RCs were overlaid to show that, from one structure to the other, the position of the center of Q_B varies almost continuously along a ~5-Å path (Lancaster et al., 1995). To make this situation even worse, the two extreme locations of Q_B are found in the two highest resolution structures presently available, one being for *Rb. sphaeroides* (Ermler et al., 1994) and the other for *Rp. viridis* (Lancaster et al., 1995). Although the binding of Q_B could be different in the RC of the two species, these discrepancies between the various structural models are more likely due, at least in part, to the fact that a large fraction of Q_B is frequently lost upon isolation of the RCs or under the conditions required for crystallization and X-ray study of the RC protein. Pending the availability of higher resolution X-ray structures and of a better parameterization of the cofactors, the determination of the details and relative strength of the bonding interactions of Q_A and Q_B with the protein relies on structural spectroscopy methods. Furthermore, X-ray studies yield an essentially static view of the RC in the neutral state and provide information neither on the light-induced structural changes accompanying the charge separation and stabilization processes nor on the geometry and bonding interactions in the semiquinone state.

Among the spectroscopic techniques that can selectively probe the bonding interactions of the quinones with the protein, light-induced FTIR difference spectroscopy appears well suited to investigate both the neutral and reduced forms of the quinones (Bagley et al., 1990; Bauscher et al., 1993; Berthomieu et al., 1990, 1992; Breton et al., 1991a–c, 1992, 1994a–c, 1995; Brudler et al., 1994; Buchanan et al., 1990, 1992; Mäntele et al., 1990; Nabedryk et al., 1990, 1991; Thibodeau et al., 1990a,b, 1992). Analyzing the light-induced Q_A^-/Q_A FTIR difference spectra of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with isotopically labeled ubiquinones, the C=O and C=C vibrational modes of Q_A were revealed for the first time and an asymmetry in the bonding interactions of the two carbonyls of Q_A could be determined (Breton et al., 1994a). FTIR investigation of RCs reconstituted with the chainless symmetrical 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone in the Q_A site has further shown that the asymmetry is not caused by the difference in the substituents at the 5- and 6-positions of the ubiquinone but rather by the different proteic environment of the two carbonyls (Breton et al., 1994b). The unambiguous assignment of the frequency of each of the two carbonyl modes of Q_A in *Rb. sphaeroides* RCs was recently achieved by site-specific isotope labeling of one or the other of the carbonyls (Breton et al., 1994c; Brudler et al., 1994).

In the present study, the issues of the degree of symmetry of the carbonyl vibrations of Q_B in its binding site and of a possible difference in the bonding interactions of the carbonyls in *Rb. sphaeroides* and *Rp. viridis* RCs are being addressed by analyzing the light-induced FTIR Q_B^-/Q_B difference spectra of RCs reconstituted with isotopically labeled ubiquinones.

MATERIALS AND METHODS

Q_B-depleted RCs from *Rb. sphaeroides* R-26 and *Rp. viridis*, prepared according to published procedures (Okamura et al., 1975; Shope & Wraight, 1985), were reconstituted under argon with excess ubiquinone as previously described (Breton et al., 1991b). The RC samples were covered with a solution containing 10 mM sodium ascorbate, 20 mM 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD), and 100 mM Tris-HCl (pH 8.0). The preparation of the isotopically labeled ubiquinones for Q_6 ^{18}O-labeled on both carbonyls and for Q_8 uniformly ^{13}C-labeled (Breton et al., 1994a; Berger et al., 1994) as well as for Q_3 selectively labeled with ^{13}C at the C_1- or the C_4-position (see inset of Figure 3, for numbering of the quinone ring) has been described previously (Breton et al., 1994c). For reconstitution with ^{18}O-labeled Q_6, the RC samples and reaction mixture were prepared in $H_2{}^{18}O$ to avoid the back-exchange of the oxygen carbonyls (Breton et al., 1994a).

Light-induced FTIR measurements were performed under single-turnover saturating laser flash excitation (530 nm, 7 ns) at 15 °C as previously reported (Breton et al., 1991b). The Q_B^-/Q_B spectra were recorded by measuring a series of three consecutive single-beam spectra (typically 128 scans each, corresponding to a recording time of 23 s) with an interval of 2 s in between. The laser flash was fired in the interval between the recording of the first and second single-beam spectra. Upon taking the ratio of these single-beam spectra, two difference spectra having a delay of 25 s between them were obtained. This procedure, which allows for an estimate of the decay of the Q_B^- state after the flash, was used to set a conservative delay between the successive series of single-beam measurements in order to prevent any contribution from the QH_2 state. These measurements were cycled typically for 6–12 h before interchanging samples. In these Q_B^-/Q_B spectra, possible contributions arising from the oxidation of the primary electron donor (P), from the cytochromes of *Rp. viridis*, from the photoreduction of the intermediary bacteriopheophytin electron acceptor, from Q_A^-/Q_A, or from oxidation of DAD and ascorbate were ruled out by comparison with the well-characterized FTIR difference spectra of the corresponding states (Breton et al., 1991a, 1992; Nabedryk et al., 1990, 1991, 1995). Using double-flash excitation of the samples, it was further verified that the signals in the Q_B^-/Q_B spectra exhibit oscillations with the binary period (Breton et al., 1991b) characteristic of this state. The extent of Q_B reconstitution, as checked by the relative amplitude of the Q_B^-/Q_B signals measured before and after reconstitution of the RCs, was greater than 75%.

RESULTS

Effect of Nonselective Labeling with ^{18}O or ^{13}C on the Q_B^-/Q_B FTIR Difference Spectra. The Q_B^-/Q_B spectrum of Q_B-depleted *Rb. sphaeroides* RCs reconstituted with unlabeled Q_6 (Figure 1a) or Q_8 (not shown) is essentially indistinguishable from that of RCs reconstituted with Q_{10} (Breton et al., 1991b). This situation is also observed for the Q_B^-/Q_B spectrum of *Rp. viridis* [Figure 2a and Breton et al. (1991b)]. In these spectra, the bands of the neutral Q_B molecule appear as negative signals while the semiquinone contributes positive bands. Additional signals arising from absorbance increases and decreases or band shifts of vibrations from the protein or from the other cofactors in response to the photoreduction of Q_B are also present in the difference spectra. When the RCs are reconstituted with Q_6 labeled with ^{18}O on both carbonyls (Figures 1b and 2b) or with uniformly ^{13}C-labeled Q_8

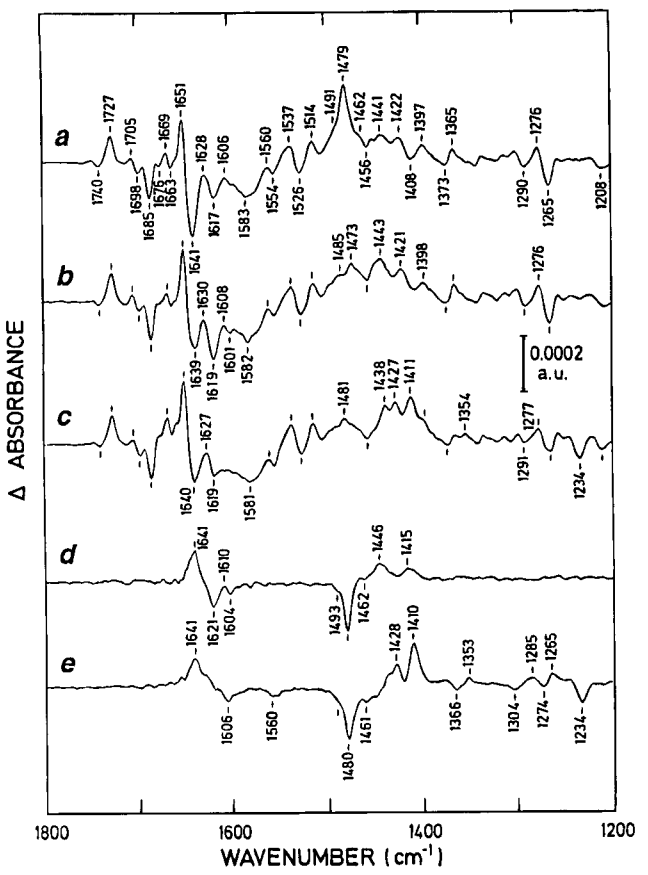

FIGURE 1: Light-induced Q_B^-/Q_B FTIR difference spectra at 15 °C of Q_B-depleted *Rb. sphaeroides* RCs reconstituted with (a) unlabeled Q_6, (b) Q_6 ^{18}O-labeled on both carbonyl groups, and (c) uniformly ^{13}C-labeled Q_8. Double-difference spectra (isotopically labeled minus unlabeled) obtained for ^{18}O-labeled Q_6 (d) and ^{13}C-labeled Q_8 (e). Average of 6−10 different pairs of samples. For each pair of spectra, the Q_B^-/Q_B spectrum obtained with RCs reconstituted with the unlabeled ubiquinone was subtracted from that obtained with RCs reconstituted with the isotopically labeled quinone. a.u., absorbance units; ~150 000 interferograms co-added; 4 cm^{-1} resolution. The frequency of the IR bands is given with an accuracy of ±1 cm^{-1}.

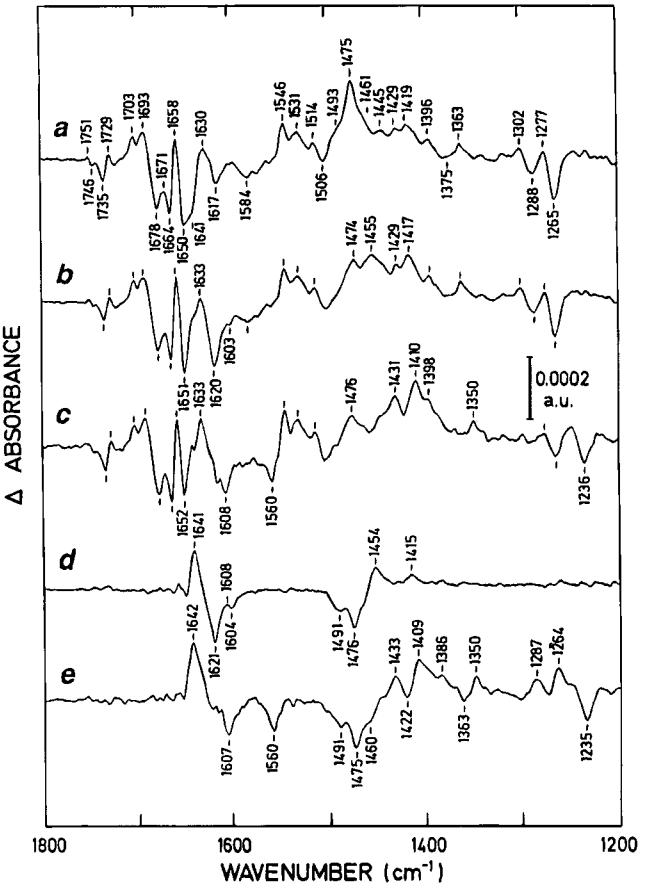

FIGURE 2: Light-induced Q_B^-/Q_B FTIR difference spectra at 15 °C of Q_B-depleted *Rp. viridis* RCs reconstituted with (a) unlabeled Q_6, (b) Q_6 ^{18}O-labeled on both carbonyl groups, and (c) uniformly ^{13}C-labeled Q_8. Double-difference spectra (isotopically labeled minus unlabeled) obtained for ^{18}O-labeled Q_6 (d) and ^{13}C-labeled Q_8 (e).

(Figures 1c and 2c), the amplitude and the position of several bands in the Q_B^-/Q_B spectra are significantly different from those found with unlabeled Q_6. These differences occur in the spectral range 1550−1670 cm^{-1}, where the C=O and C=C vibrations of the neutral quinones are found, and between 1400 and 1500 cm^{-1}, where the C···O and C···C modes of the semiquinone are expected to contribute. A negative band at 1641 cm^{-1} in the Q_B^-/Q_B spectra of unlabeled Q_6 (Figures 1a and 2a) decreases strongly in amplitude upon both types of labeling. In the case of ^{18}O labeling, this is accompanied by an increase of the amplitude of a band at ~1620 cm^{-1} (Figures 1b and 2b). The large positive band at 1479 cm^{-1} in *Rb. sphaeroides* (Figure 1a) or at 1475 cm^{-1} in *Rp. viridis* (Figure 2a) also decreases in amplitude upon labeling while positive bands increase in amplitude at lower frequency. In the case of ^{13}C labeling, several additional bands appear affected, notably below 1400 cm^{-1}. In contrast to these bands that respond to the isotope substitution and thus correspond to quinone modes, many bands in these spectra appear unaffected by the labeling, notably in the region above 1650 cm^{-1} and in the 1550−1500-cm^{-1} frequency range. In the case of uniform ^{13}C labeling, for which all of the quinone vibrations (i.e., ring, carbonyls, methyl, methoxys, and isoprenoid chain) are perturbed by the labeling, these unaffected bands cannot originate from the quinone and thus pertain to the protein.

Isotope-sensitive vibrations from the quinone itself in the Q_B^-/Q_B spectra can be separated from those of the protein by calculating the double-difference spectrum between a pair of Q_B^-/Q_B spectra recorded with RCs reconstituted with isotopically labeled and unlabeled quinones (Breton et al., 1994a−c). Such double-difference spectra are shown for ^{18}O (Figures 1d and 2d) and ^{13}C labeling (Figures 1e and 2e). In these double-difference (isotopically labeled minus unlabeled) spectra, the IR bands of the neutral unlabeled Q_B appear with a positive sign while the downshifted bands of the labeled quinone exhibit a negative sign. A reverse situation is found for the semiquinone bands. Only those vibrations of the quinone *in vivo* that are affected by the labeling will contribute.[2] The decrease of intensity of the vibrational modes upon isotope labeling as well as the overlap of the positive and negative bands can lead to an apparent cancellation of some of the bands (Breton et al.,

[2] The protein bands and protein−quinone interactions detected in the Q_B^-/Q_B spectra are not affected by incubation of the RCs in $H_2{}^{18}O$. This conclusion derives from the observation of an identical pattern for the bands corresponding to the unlabeled quinone when $H_2{}^{18}O$ is used for the incubation (Figures 1d and 2d) instead of $H_2{}^{16}O$ (Figures 1e and 2e). Additional evidence has also been obtained upon double-labeling of the uniformly or selectively ^{13}C-labeled ubiquinones with ^{18}O on both carbonyls.

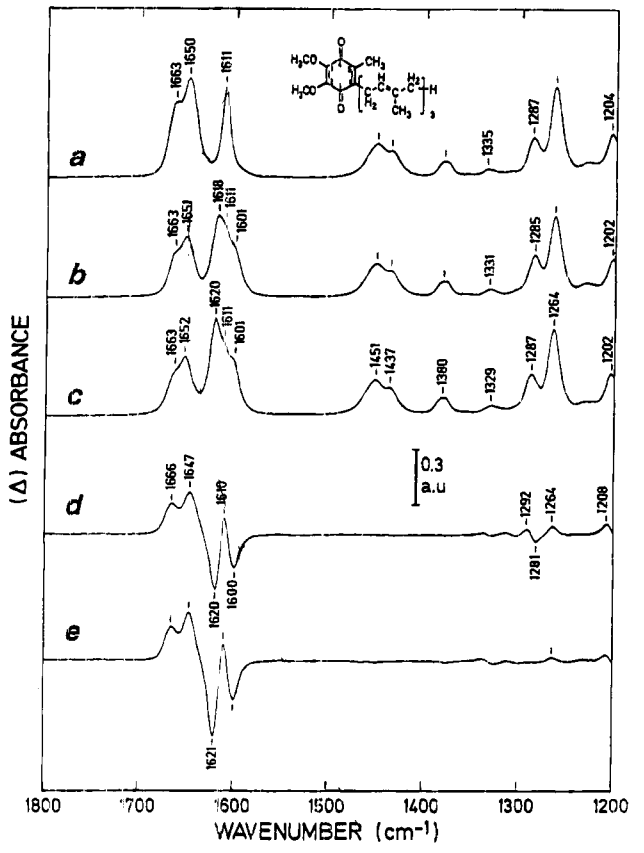

FIGURE 3: IR absorption spectra of films of (a) unlabeled Q_3, (b) $^{13}C_1$-labeled Q_3, and (c) $^{13}C_4$-labeled Q_3. The individual spectra were normalized as described in the text. Difference spectra between the unlabeled Q_3 and (d) $^{13}C_1$-labeled Q_3 or (e) $^{13}C_4$-labeled Q_3. Inset: Structural formula of ubiquinone-3 (Q_3; 2,3-dimethoxy-5-methyl-6-triprenyl-1,4-benzoquinone).

1994a–c). Upon labeling either with ^{18}O on both carbonyls of Q_B (Figures 1d and 2d) or uniformly with ^{13}C (Figures 1e and 2e), an unsplit positive band of large amplitude is observed at 1641 cm^{-1} for both *Rb. sphaeroides* (Figure 1d,e) and *Rp. viridis* (Figure 2d,e). For both species, this band appears to shift to 1621 cm^{-1} for ^{18}O labeling and to ~1607 cm^{-1} for ^{13}C labeling. Small negative bands are observed at 1604 cm^{-1} and at 1560 cm^{-1} upon ^{18}O and ^{13}C labeling, respectively. In this frequency range and for each type of labeling, the closeness of the double-difference spectra for the RCs of the two species is striking. Although less pronounced than for the vibrations of the neutral Q_B, the double-difference spectra also display a marked analogy between *Rb. sphaeroides* and *Rp. viridis* for the semiquinone vibrations. Together with the disappearance of the main vibration of the unlabeled semiquinone at 1479 (*Rb. sphaeroides*) or 1475 cm^{-1} (*Rp. viridis*), two positive bands appear at ~1450 and 1415 cm^{-1} upon ^{18}O labeling and at ~1430 and 1410 cm^{-1} upon ^{13}C labeling. Although the semiquinone bands appear sharper in *Rb. sphaeroides* than in *Rp. viridis* and a broad negative shoulder at ~1492 cm^{-1} is much more pronounced in *Rp. viridis* than in *Rb. sphaeroides*, the relative intensities of the positive bands in the double-difference spectra appear more dependent on the type of label than on the RC species.

Effect of ^{13}C Selective Labeling of the Carbonyls on the Q_B^-/Q_B FTIR Difference Spectra. The absorption spectra (Figure 3a–c) of films of the isolated Q_3 molecules used for the reconstitution have been normalized on the δCH_2 and δCH_3 vibrations of the chain and of the methoxy groups giving rise to the bands at 1451 and 1437 cm^{-1} (Bellamy, 1980; Breton et al., 1994c), which appear essentially unaffected by the isotopic labeling. The ~50% amplitude decrease of the carbonyl bands at 1663 and 1650 cm^{-1} is accompanied by the appearance of a new band at 1620–1618 cm^{-1} and by the shift of a fraction of the C=C band from 1611 to 1601 cm^{-1}. The C=C band is identically affected by the labeling at C_1 or at C_4, as shown by the close equivalence of the difference spectra (unlabeled minus labeled Q_3) for the two positions (Figure 3d,e).

For both *Rb. sphaeroides* (Figure 4a) and *Rp. viridis* (Figure 5a), the Q_B^-/Q_B spectra of Q_B-depleted RCs reconstituted with unlabeled Q_3 are essentially indistinguishable from those recorded with Q_6, Q_8, or Q_{10}. When the RCs are reconstituted with selectively ^{13}C-labeled Q_3, pronounced effects are seen, notably on the 1641-cm^{-1} band, which decreases in amplitude for both $^{13}C_1$ and $^{13}C_4$ labeling. A single negative band at 1618 cm^{-1} is seen for $^{13}C_4$ labeling (Figures 4c and 5c), while two negative bands at 1619–1622 and 1599–1606 cm^{-1} are observed for $^{13}C_1$ labeling (Figures 4b and 5b), demonstrating some asymmetry in the bonding interactions of the neutral Q_B with the protein. In the semiquinone absorption region, positive bands at ~1439 and ~1444 cm^{-1} increase in amplitude in the Q_B^-/Q_B spectra of *Rb. sphaeroides* (Figure 4b,c) and *Rp. viridis* (Figure 5b,c), respectively. Differences in the isotope effects are also observed, notably upon $^{13}C_1$ labeling with the pronounced amplitude increase of a band at 1489 cm^{-1}, suggesting some asymmetry in the binding of the quinone also in the reduced state.

These effects are best revealed in the double-difference spectra, which show an identical frequency of the unlabeled C_1=O and C_4=O modes (1641 cm^{-1}) of Q_B for both *Rb. sphaeroides* (Figure 4d,e) and *Rp. viridis* (Figure 5d,e). On the other hand, an inequivalence of the quinone modes for labeling at the two positions is detected in the 1630–1580-cm^{-1} frequency range of the C=C vibrations, where the downshift to 1600 cm^{-1} of a mode absorbing at 1610–1615 cm^{-1} is observed only for labeling at C_1. This inequivalence is clearly seen when the effects of selective labeling on the C=C vibrations of the isolated quinones (Figure 3d,e) are compared to the corresponding effects in the RCs (Figures 4d,e and 5d,e). The pattern of two negative bands at 1620–1621 and 1600 cm^{-1} with a positive band in between at 1610 cm^{-1} seen in the difference spectra of the isolated Q_3 is recognized for labeling at the 1-position *in vivo* (with negative bands at 1620 and 1600 cm^{-1} in *Rb. sphaeroides* or at 1624 and 1601 cm^{-1} in *Rp. viridis* and a positive one at 1610 cm^{-1} in *Rb. sphaeroides* or 1615 cm^{-1} in *Rp. viridis*) but is missing for labeling at the 4-position. In this case, a single negative band at 1618–1619 cm^{-1} is observed (Figures 4e and 5e). Similarly, an inequivalence is also evident in the semiquinone absorption range with increases or decreases of the absorption around 1490 cm^{-1} upon labeling at the 1- or the 4-position, respectively. The amplitude of a mode at 1410–1415 cm^{-1} is found to be larger for labeling at the 1- than at the 4-position for the Q_B^-/Q_B spectra recorded with the RCs of both species.

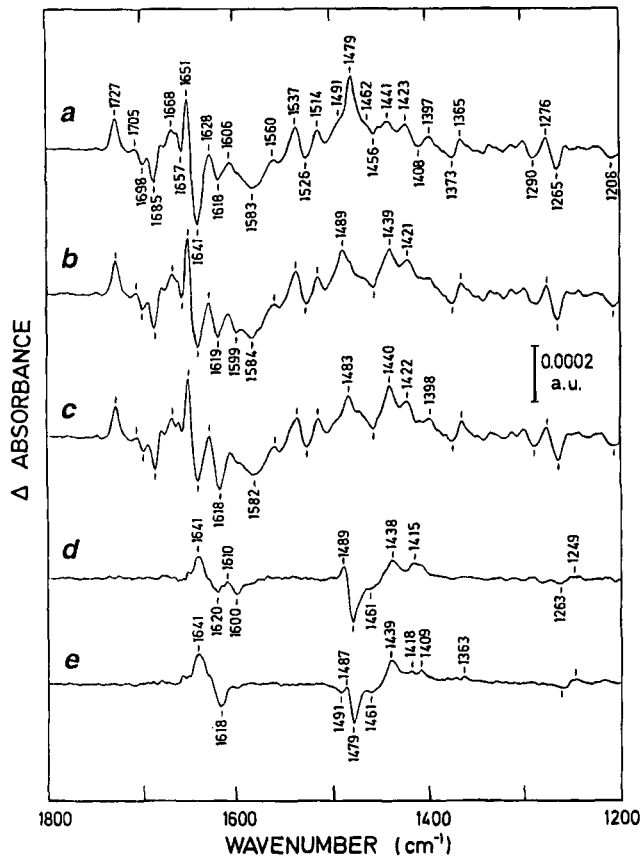

FIGURE 4: Light-induced Q_B^-/Q_B FTIR difference spectra at 15 °C of Q_B-depleted *Rb. sphaeroides* RCs reconstituted with (a) unlabeled Q_3, (b) $^{13}C_1$-labeled Q_3, and (c) $^{13}C_4$-labeled Q_3. Double-difference spectra (isotopically labeled minus unlabeled) obtained for $^{13}C_1$-labeled Q_3 (d) and $^{13}C_4$-labeled Q_3 (e).

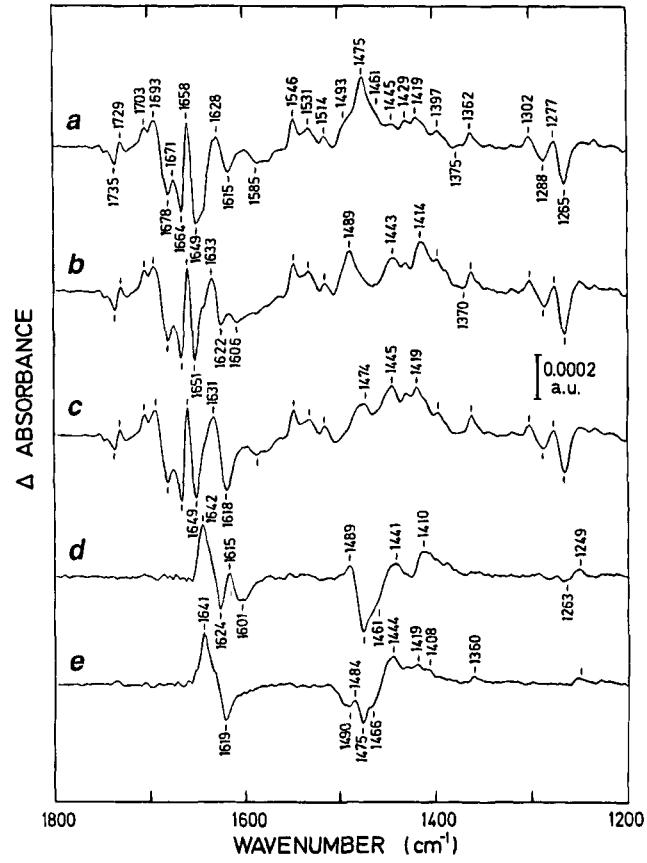

FIGURE 5: Light-induced Q_B^-/Q_B FTIR difference spectra at 15 °C of Q_B-depleted *Rp. viridis* RCs reconstituted with (a) unlabeled Q_3, (b) $^{13}C_1$-labeled Q_3, and (c) $^{13}C_4$-labeled Q_3. Double-difference spectra (isotopically labeled minus unlabeled) obtained for $^{13}C_1$-labeled Q_3 (d) and $^{13}C_4$-labeled Q_3 (e).

DISCUSSION

The light-induced FTIR Q_B^-/Q_B difference spectra of Q_B-depleted RCs of *Rb. sphaeroides* and *Rp. viridis* reconstituted with several isotopically labeled ubiquinones reported here reveal pronounced effects reflecting large frequency shifts of modes of both the neutral and the anion forms of Q_B. For the vibrations of the neutral quinones, these isotopic shifts appear comparable, although not identical, to those observed upon labeling of the isolated quinones [Breton et al. (1994a) and Figure 3]. In addition, several of the Q_B^-/Q_B bands that are not affected by the isotope substitutions, notably in the case of reconstitution with uniformly ^{13}C-labeled Q_8, can be unambiguously assigned to modes corresponding to stable structural rearrangements of the protein which accompany the photoreduction of Q_B. As previously noticed for Q_A photoreduction (Breton et al., 1994a), the amplitude of the protein bands in the Q_B^-/Q_B spectra is roughly comparable to that of the quinone modes, suggesting similar overall perturbation of the protein and of the quinone. While these changes are obviously localized on a small number of bonds for the quinone, they are probably distributed over a much larger number of bonds at the level of the protein as suggested by molecular dynamics simulations (Nonella & Schulten, 1991). The large differential signals arising from the protein, notably in the 1650−1700-cm^{-1} frequency range, represent a serious difficulty when the double-difference spectra are generated and analyzed (Breton et al., 1995). A specific problem is encountered around 1650−1670 cm^{-1}, which represents the most variable region of the Q_B^-/Q_B difference spectra (compare, e.g., Figures 1a and 4a or 2a and 5a). The FTIR signals in this frequency range have been observed to vary slightly from sample to sample even when the very same treatment is applied to a given batch of RCs. They notably appear sensitive to "aging" effects of the sample. In the present study, this problem has been overcome by averaging a large number of individual spectra taken (i) with different samples and (ii) for each sample, at various delay times after preparation and over a 2−3-day period. On the other hand and as previously noticed for the Q_A^-/Q_A spectra of *Rb. sphaeroides* RCs reconstituted with different ubiquinones (Breton et al., 1994a,c), varying between 3 and 10 the number of isoprene units of the chain appears not to affect the Q_B^-/Q_B spectra. As the Q_B^-/Q_B difference spectra recorded with Q_0 in the Q_B site of *Rb. sphaeroides* and *Rp. viridis* (Breton et al., 1991b) differ significantly from those reported here, it can be surmised that only the very first units of the chain may play a role in the proper anchoring of the quinone head group to the Q_B site. This observation agrees with the results of binding affinity studies showing (i) identical affinities of Q_3 and Q_{10} at the Q_B site and (ii) that the first isoprene unit is providing most of the specificity of the anchoring of the quinone to its protein site (McComb et al., 1990; Warnke et al., 1994).

Isotope Effects on the Quinone Vibrations in Vitro. The origin of the splitting of the carbonyl bands of the isolated ubiquinones and the question of the extent of coupling of the C=O vibrations have been addressed in previous

publications in this series (Breton et al., 1994a–c). As the methoxy groups of 2,3-dimethoxy-1,4-benzoquinones can adopt two different conformations in solution, the splitting of the carbonyl band is best explained by a differential inductive effect of each methoxy group on the proximal C=O bond. In this scheme, one of the O–CH_3 bonds, oriented in the quinone plane and pointing away from the proximal carbonyl oxygen, is responsible for the high-energy C=O band at 1663 cm^{-1}, while the O–CH_3 bond of the second methoxy is oriented at 120° from the quinone plane and gives rise to the C=O band at 1650 cm^{-1} (Breton et al., 1994c; Burie, 1994). This geometry of the methoxy groups being essentially independent of the nature of the substituents at the 5- and 6-positions of the quinone ring, two different conformers are expected for nonsymmetrical ubiquinones. For a given carbonyl, e.g., C_1, two IR C=O bands corresponding to the in-plane and out-of-plane orientations of the proximal methoxy group are observed at 1663 and 1650 cm^{-1}, respectively, for the unlabeled quinone and both bands downshift upon selective labeling. As the same property also applies to the C_4 carbonyl, it can be understood that only ~50% of the 1663- and 1650-cm^{-1} bands shift for each selective label. The slight differences observed in the peak positions and relative amplitudes of the $^{13}C_1$=O (1618 cm^{-1}) and $^{13}C_4$=O (1620 cm^{-1}) bands (Figure 3b,c) are probably related to the coupling of these modes to vibrations involving the different substituents at the 5- and 6-positions (Meyerson, 1985).

The question of the degree of coupling of the vibrations of the two C=O groups and notably the problem of the influence of a possible coupling of these vibrational modes on the IR frequency of the carbonyls has been examined both by partial ^{18}O labeling of the carbonyls of symmetrical quinones (Breton et al., 1994b) and by selective ^{13}C labeling of Q_3 (Breton et al., 1994c). In both cases, it was concluded that such a coupling, if present, has only minor spectroscopic consequences on the frequency of the carbonyl bands and that the two C=O bands of the ubiquinones can be essentially considered as corresponding to the vibrational modes of each individual C=O group.

The C=C vibrations of ubiquinone *in vitro*, observed at 1611 cm^{-1} (Figure 3a), are partially coupled to the C=O modes as shown by the isotopic shifts of 5 and 10 cm^{-1} found upon labeling of the carbonyl oxygen atoms (Breton et al., 1994a) and of the C_1 or C_4 atoms (Figure 3b,c), respectively. This coupling is symmetrical as the C=C vibrations are affected in the same way by labeling at the 1- or the 4-position (Figure 3d,e). It is worth noting that only approximately half of the C=C band shifts to 1601 cm^{-1} upon $^{13}C_1$ or $^{13}C_4$ labeling while all of the band shifts to 1596 cm^{-1} upon selective labeling of the C_5 or C_6 atoms (Brudler et al., 1994) and to 1554 cm^{-1} upon uniform ^{13}C labeling (Breton et al., 1994a).

Vibrations of Neutral Q_B in Rb. sphaeroides and Rp. viridis. The single positive band of large amplitude observed at 1641 cm^{-1} in the double-difference spectra obtained upon reconstitution of the RCs of both species with nonspecifically labeled quinones falls in the typical frequency range of the C=O vibrations of quinones in solution although it is downshifted by 10–20 cm^{-1} compared to that of isolated ubiquinone. This band shifts to 1621 or ~1607 cm^{-1} upon ^{18}O labeling of the carbonyls or uniform ^{13}C labeling, respectively. The isotopic shift of 20 cm^{-1} found upon ^{18}O labeling is smaller than that (31 ± 3 cm^{-1}) observed for Q_6 in solution and the ~34-cm^{-1} shift upon uniform ^{13}C labeling is also smaller than that (42 ± 1 cm^{-1}) observed *in vitro* (Breton et al., 1994a). The corresponding calculated shifts for a pure C=O stretching mode are of 40 and 37 cm^{-1}, respectively. The downshifts of the ubiquinone vibrational modes in the Q_B site are thus consistent with an assignment of the 1641-cm^{-1} band to a C=O mode significantly coupled to the C=C mode as previously found for the C=O modes of Q_A (Breton et al., 1994a,c). The absence of a splitting of the 1641-cm^{-1} band indicates either an identical frequency for both carbonyls of Q_B or the contribution of only one of the two C=O groups in the spectra. The double-difference spectra obtained with the selectively labeled Q_3 also show that for both isotopomers a positive band of comparable amplitude at ~1641 cm^{-1} is downshifted by 22 ± 1 cm^{-1} for *Rb. sphaeroides* and by 20 ± 2 cm^{-1} for *Rp. viridis*. This demonstrates that both carbonyls of Q_B do indeed contribute similarly to the 1641-cm^{-1} band. In this case also, the isotopic shifts observed *in vivo* are significantly smaller than that (~32 cm^{-1}) observed for the isolated Q_3 molecules.

The presence of a small derivative signal positive at 1609 ± 1 cm^{-1} and negative at 1604 cm^{-1} in the double-difference spectra obtained upon ^{18}O labeling of both carbonyls of Q_B (Figures 1d and 2d) as well as the appearance of a negative band at 1560 cm^{-1} upon uniform ^{13}C labeling (Figures 1e and 2e) provides clear signatures for a C=C mode in the Q_B state. The C=C mode of the unlabeled quinone, although difficult to locate precisely in the double-difference spectra due to overlap with the downshifted C=O mode, appears to contribute to the negative band at 1615–1618 cm^{-1} in the Q_B^-/Q_B spectra recorded with unlabeled quinones. A differential signal positive at 1610–1615 cm^{-1} and negative at ~1600 cm^{-1} is observed upon $^{13}C_1$ labeling (Figures 4d and 5d) but is absent upon $^{13}C_4$ labeling (Figures 4e and 5e). A strikingly similar signal is also observed at 1610/1600 cm^{-1} in the two difference spectra calculated for the isotope effect on the isolated quinones (Figures 3d,e). In this case, the differential signal can be unambiguously ascribed to the downshift of the C=C mode upon labeling. The observation that this differential signal is present *in vitro* for both labeling positions of the quinone while it is only observed for labeling at C_1 *in vivo* indicates a very peculiar asymmetry of the two carbonyls in the Q_B site. It appears that the Q_B C=C mode absorbing at ~1617 cm^{-1} involves motion of the C_1 atom but not of the C_4 atom, in contrast to the 1611-cm^{-1} C=C band of ubiquinone in solution for which the motion of both atoms is involved. The identical extent of perturbation of the frequency of the two carbonyls of Q_B upon binding to the RC is thus contrasted by a strong inequivalence of their coupling to the C=C mode.

On the basis of a comparison of the Q_B^-/Q_B difference spectra obtained with RCs of both *Rb. sphaeroides* and *Rp. viridis* reconstituted either with Q_{10} or with Q_0, it has been proposed that the Q_B carbonyl vibrations contribute to the band at 1640 cm^{-1} while the C=C vibrations absorb at 1616–1618 cm^{-1} (Breton et al., 1991b). Furthermore, the relative amplitude of bands at 1636 and 1618 cm^{-1} in the $P^+Q_B^-/PQ_B$ spectrum of *Rb. sphaeroides* RCs has been observed to vary depending upon the isotope composition of the Q_{10} molecule used to reconstitute the Q_B site (Bagley et al., 1990). In addition a prominent positive band at ~1640 cm^{-1} also appears in the $Q_A^-Q_B/Q_AQ_B^-$ double-difference

spectra obtained by a time-resolved (rapid-scan) FTIR technique for both *Rb. sphaeroides* (Thibodeau et al., 1990a,b) and *Rp. viridis* (Thibodeau et al., 1992). In contrast, none of the Q_B or Q_B^- bands are seen in a previously reported $Q_A^-Q_B/Q_AQ_B^-$ double-difference spectrum calculated from P^+Q^-/PQ spectra of *Rp. viridis* (Buchanan et al., 1992), indicating that these authors most probably failed to generate significant amounts of the $P^+Q_B^-/PQ_B$ state.

Semiquinone Vibrations in Q_B^-. In the complex region of absorption of the C:··O and C:··C modes of Q_B^-, isotope labeling leads to a large decrease of the amplitude of the main anion bands at 1479 and 1475 cm^{-1} in the Q_B^-/Q_B spectra of *Rb. sphaeroides* and *Rp. viridis*, respectively. By comparison with spectra of electrochemically generated semiquinone model compounds in various solvents (Bauscher et al., 1990; Bauscher & Mäntele, 1992), this main band was previously assigned to a predominantly C:··O stretch in Q_B^- (Breton et al., 1991b). The double-difference spectra upon ^{18}O labeling of the carbonyls (Figures 1d and 2d) indicate that this mode is downshifted by about 34 and 22 cm^{-1} in *Rb. sphaeroides* and *Rp. viridis*, respectively, consistent with such an assignment. The downshift of this band upon uniform ^{13}C labeling is 52 cm^{-1} for *Rb. sphaeroides* and 42 cm^{-1} for *Rp. viridis* (Figures 1e and 2e), showing that this mode is also strongly coupled to the C:··C modes, as previously inferred from *ab initio* calculations (Chipman & Prebenda, 1986). The positive bands around 1410−1415 cm^{-1} in these double-difference spectra are in part ascribed to bands arising from the downshift of C:··C modes underlying the low-energy side of the main anion band at 1480−1475 cm^{-1} and giving rise to the shoulder at ~1461 cm^{-1} in the Q_B^-/Q_B spectra for the unlabeled quinone. Enhancement of C:··C modes absorbing at lower frequency and partially masked by the downshifted C:··O mode probably also contributes to the 1410−1415-cm^{-1} bands. It should, however, be stressed that, in the absence of Q_B^-/Q_B spectra for ubiquinone selectively labeled on the other carbon atoms of the ring, of *in vitro* anion spectra of selectively labeled quinones, and of normal-mode analysis of the anion spectra, these tentative assignments for Q_B^- should only be considered as provisional.

As previously noticed for the C=O vibrations of the neutral Q_B, the absence of splitting of the main C:··O mode indicates either a symmetrical bonding interaction of the two carbonyls or that only one carbonyl contributes to the FTIR spectrum. Still another possibility is that the two C:··O modes are strongly coupled and give rise to a single band. In the case of the selective labeling of one or the other of the two carbonyls, the double-difference spectra reveal a clear inequivalence of the vibrations around 1490 and 1415 cm^{-1}. This is taken to indicate that, although it is probable that each of the semiquinone modes involves the motion of many atoms of the quinone ring, this mixing of the modes does not prevent an intrinsic asymmetry to be revealed.

A band is present at ~1490 cm^{-1} in all the double-difference spectra, although it only appears as a negative shoulder in the spectra obtained with nonselectively labeled quinones. It is usually more pronounced for *Rp. viridis* than for *Rb. sphaeroides*. This band exhibits very peculiar behavior upon selective labeling, increasing in intensity upon $^{13}C_1$ labeling (Figures 4d and 5d) and decreasing upon $^{13}C_4$ labeling (Figures 4e and 5e). A very similar behavior has been observed for a band at 1484 cm^{-1} in the Q_A^-/Q_A spectra of *Rb. sphaeroides* RCs. This band has been proposed to contain predominantly C:··C modes involving both the quinone ring and the substituents (Breton et al., 1994a−c). The comparable behavior of this band in the Q_B^-/Q_B spectra favors the same assignment.

The main anion mode at 1479 cm^{-1} in *Rb. sphaeroides* is downshifted to almost the same frequency (1438−1439 cm^{-1}) upon selective labeling at either the 1- or the 4-position. This, together with the unsplit character of the 1479-cm^{-1} band, show that the perturbation of the carbonyl frequency of the semiquinone state due to the interactions with the protein is the same for the two C:··O groups. A similar conclusion also applies to the 1475-cm^{-1} Q_B^- band in *Rp. viridis*. However, an inequivalence is seen in the amplitude of the modes, which are downshifted to ~1410−1420 cm^{-1}. For the RCs of both species, the amplitude of these bands is significantly larger for labeling at the 1-position than at the 4-position. As these bands are likely to represent predominantly C:··C modes, we conclude that, similarly to the neutral Q_B state, the asymmetric binding perturbs the C:··C more than the C:··O vibrations.

The positive anion band at 1365 cm^{-1} and the associated negative band at 1373 cm^{-1} in the Q_B^-/Q_B spectra of *Rb. sphaeroides* correspond well to Q_A^-/Q_A signals that have been assigned to δCH_3 vibrations of the methyl group at the 5-position of the ring (Breton et al., 1994b,c). Consistent with this assignment, these modes are not shifted by the labeling of the two oxygen carbonyls (Figures 1a,b,d and 2a,b,d) but are significantly affected by the uniform ^{13}C labeling (Figures 1c,e and 2c,e). The double-difference spectra for the selective labeling of each carbonyl (Figures 4d,e and 5d,e) provide an indication that the labeling at the 4-position enhances the anion mode at 1362−1365 cm^{-1} slightly more than labeling at the 1-position. A similar selectivity in the enhancement of a very similar mode upon labeling at the 4-position is also visible in the corresponding double-difference spectra for the Q_A^-/Q_A state (Breton et al., 1994c). These observations, together with the smaller frequency span between the negative and the positive peaks in the Q_B^-/Q_B than in the Q_A^-/Q_A spectra, suggest that this set of bands may provide a useful marker to investigate the conformational constraints of the methyl group in the Q_A and Q_B binding sites. In this respect, selective labeling of the methyl at the 5′-position would be useful.

Interaction of Q_B and Q_B^- with the Protein. The present study demonstrates that the C_1 and C_4 carbonyls of Q_B contribute equally to the band at 1641 cm^{-1}. Taken together with the finding that the two C=O groups of ubiquinones both in solution (Breton et al., 1994b) and in the Q_A site of *Rb. sphaeroides* (Breton et al., 1994c) behave spectroscopically like isolated vibrators, this observation shows that each carbonyl of Q_B is engaged in highly comparable interactions with the protein at the binding site. Furthermore, the observation of an identical frequency for the C=O vibrations of Q_B in the RCs of *Rb. sphaeroides* and of *Rp. viridis* strongly suggests comparable interactions in both RCs. Compared to the C=O frequency of ubiquinone *in vitro*, the band at 1641 cm^{-1} in the RCs is downshifted by either 9 or 22 cm^{-1}, depending on the actual conformation of the methoxy groups in the binding site. Assuming that no other factor affects the comparison between the *in vivo* and *in vitro* data, these shifts would be indicative of weak to moderate

hydrogen-bonding interactions of the carbonyls of Q_B with the protein.

In the Q_B^- semiquinone state, the C_1···O and C_4···O carbonyls also appear degenerate and are responsible for the main anion bands at 1479 and 1475 cm^{-1} in the Q_B^-/Q_B spectra of *Rb. sphaeroides* and *Rp. viridis*, respectively. Compared to the frequency of the main C···O mode of Q_{10} observed at 1488 and 1482 cm^{-1} in the non-hydrogen-bonding solvents THF and CH_2Cl_2, respectively (Bauscher et al., 1990; Bauscher & Mäntele, 1992), the small frequency downshifts (3−13 cm^{-1}) measured *in vivo* also suggest weak bonding interactions in the reduced state of Q_B. At a glance, this result appears in contradiction with literature data suggesting much stronger interactions for Q_B^- than for Q_B (Diner et al., 1984). This point will be addressed below.

The most unexpected result emerging from the present study concerns the very different behavior of the C=C vibrations of Q_B and Q_B^- upon selective ^{13}C labeling of each carbonyl. A trivial explanation would be that this effect is not caused by the binding of the quinone to the protein but rather is the mere consequence of the intrinsic asymmetry of the ubiquinone resulting from the difference of the substituents at the 5- and 6-positions. Indeed, slight differences have already been noticed in the absorption spectra of the isolated $^{13}C_1$- and $^{13}C_4$-labeled Q_3 (Figure 3b,c). That this is not the cause of the effect observed *in vivo* can be demonstrated by comparing the differences between the absorption spectra of the unlabeled and labeled Q_3 (Figure 3d,e) to the corresponding *in vivo* double-difference spectra (Figures 4d,e and 5d,e). The two difference spectra calculated for the isolated Q_3 (Figure 3d,e) exhibit three positive bands at 1666, 1647, and 1610 cm^{-1} and two negative bands at 1621−1620 and 1600 cm^{-1}. Thus, the 1610/1600-cm^{-1} differential signal describing the shift of the C=C mode of Q_3 *in vitro* upon selective $^{13}C_1$ labeling has the same shape and almost the same amplitude as that observed for $^{13}C_4$ labeling. In sharp contrast, the equivalent differential signal at 1610/1600 cm^{-1} for *Rb. sphaeroides* (Figure 4d) and at 1615/1601 cm^{-1} for *Rp. viridis* (Figure 5d) describing the shift of the C=C mode of Q_3 in the Q_B site is only detected upon selective $^{13}C_1$ labeling and is essentially absent for $^{13}C_4$ labeling. This comparison of the isotopic shifts observed *in vivo* and *in vitro* shows that the protein at the Q_B site imparts a specific configuration to the quinone so that the C_4 atom is specifically perturbed compared to the isolated molecule. In contrast, the C_1 atom of Q_B behaves as it does for Q_3 *in vitro*.

The reasons for this clear differential effect of the protein on C_1 and C_4 in the Q_B site while the vibrational frequency of the two C=O groups is the same are still obscure. It is, however, striking that in the Q_A site of *Rb. sphaeroides* the C_4 atom of the ubiquinone is also dramatically perturbed, while the C_1 atom behaves essentially as in the isolated molecule. In this case, the FTIR results have been interpreted in terms of a very strong hydrogen bond to the C_4=O group, leading to a large perturbation of the C=C mode coupled to the C_4=O mode, and of a C_1=O group essentially free from interaction with the protein. While the strong hydrogen bond at the C_4=O carbonyl of Q_A has been taken as responsible for the large effect on the C=C vibrations coupled to the C_4=O mode (Breton et al., 1994c), this cannot be the reason for the differential effect seen on the C=C band of Q_B. In order to explain this effect of the protein on the differential coupling of the C=C mode of Q_B to vibrations involving displacements of the C_1 and C_4 atoms, another class of possibilities would involve the interactions of the peripheral substituents with the protein at the binding site. In this respect, it is worthy of note that the response of the 1611-cm^{-1} C=C band of the isolated ubiquinone to isotope labeling is quite complex, indicating that the mode responsible for this band involves the motion of many of the quinone atoms. The shifts of this band in response to various labels were described in a previous section. In addition, this band is unaffected by ^{13}C labeling of the methyl at the 5′-position (van Liemt, 1994), while it downshifts by 2 and 4 cm^{-1} upon ^{18}O labeling of both methoxy groups and uniform ^2H labeling, respectively (J.B., E.N., and C.B., unpublished results). This last observation is specially worthy of interest as it shows that the 1611-cm^{-1} C=C mode can be affected by changing the mass of atoms that are not part of the quinone ring. It can thus be surmised that a specific anchoring of the 5′-methyl and/or of the methoxy group at C_3 into a tight binding niche could perturb the C=C mode coupled to the C_4=O mode much more than that coupled to the C_1=O mode provided the chain and the other methoxy group are less constrained.

An indication that the 5′-methyl group is in a more constrained environment in the Q_B site than in the Q_A site is provided by the comparison of the 1365/1373-cm^{-1} differential signal in the Q_B^-/Q_B spectra of *Rb. sphaeroides* (Figures 1a and 4a) to the 1356/1371 and 1355/1372-cm^{-1} signals in the Q_A^-/Q_A spectra of *Rb. sphaeroides* RCs reconstituted with Q_6 and duroquinone (DQ; 2,3,5,6-tetramethyl-1,4-benzoquinone), respectively. These signals have been assigned to the effect of quinone photoreduction on the δCH_3 vibration of the methyl group(s) directly attached to the quinone ring (Breton et al., 1994b). Electrochemical reduction of DQ in acetonitrile (Bausher, 1991) reveals the same differential signal at 1356/1373 cm^{-1}. Thus, the 16 ± 1 cm^{-1} downshift of this vibration upon photoreduction of Q_6 or DQ in the Q_A site of *Rb. sphaeroides* is equivalent to that observed for electrochemical reduction of DQ in solution and is about twice as large as the shift found for photoreduction of ubiquinone in the Q_B site of both *Rb. sphaeroides* and *Rp. viridis*.

The possibility that the methoxy groups are directly involved in the binding of Q_B and in the conformational changes accompanying its photoreduction appears likely in view of the notorious importance of the geometry of these substituents in tuning the redox properties of ubiquinones (Prince et al., 1983, 1988; McComb et al, 1990; Robinson & Khan, 1990; Nonella & Schulten, 1991; Burie, 1994). More direct evidence for a different behavior of the methoxy groups in the Q_A and Q_B sites can be found in a comparison of the effect of selective ^{13}C labeling of the carbonyls on the bands assigned to the methoxy vibrations in the Q_A^-/Q_A and Q_B^-/Q_B spectra of *Rb. sphaeroides* (Breton et al., 1991b, 1994a). In the isolated molecule, these bands are found at 1287 and 1264 cm^{-1} and their downshift by 22 and 29 cm^{-1} upon uniform ^{13}C labeling is close to that (28 cm^{-1}) expected for a pure mode (Breton et al., 1994a). These bands are essentially unaffected by ^{18}O labeling of the carbonyls. They are only slightly affected by the selective labeling of the C_1 and C_4 atoms (Figure 3), and this small perturbation is larger when the ^{13}C label is at the 1- than at the 4-position. This differential effect of selective C_1 and C_4 labeling on each of

the methoxy group *in vitro* can be analyzed by calculating the differences between the absorption spectra of the unlabeled and selectively ^{13}C-labeled Q_3 (Figure 3). In the methoxy absorption region, the ^{12}C minus $^{13}C_1$ spectrum (Figure 3d) for the isolated Q_3 exhibits two positive bands at 1292 and 1264 cm^{-1} with a negative band in between at 1281 cm^{-1} while the ^{12}C minus $^{13}C_4$ spectrum (Figure 3e) shows only one positive band of much smaller amplitude at 1264 cm^{-1}. These difference spectra describing the effect of selective $^{13}C{=}O$ labeling on the proximal methoxy group of isolated Q_3 closely match a set of features observed at almost the same frequencies, and only for the $^{13}C_1$-labeled Q_3, in the corresponding double-difference spectra for photoreduction of Q_3 in the Q_A site of *Rb. sphaeroides* (Breton et al., 1994c). In contrast, the corresponding double-difference spectra for the photoreduction of Q_B do not show these features and are characterized instead by a single differential signal at ~1249/1263 cm^{-1} (Figures 4d,e and 5d,e). This finding is taken to indicate a different conformation of the methoxy groups in the Q_A and Q_B sites of *Rb. sphaeroides* as well as the same conformation of these groups in the Q_B site of *Rb. sphaeroides* and *Rp. viridis*. Indeed, the conformation of the methoxy groups in the Q_A binding site of *Rb. sphaeroides* was proposed to be essentially equivalent to that of ubiquinone in solution (Breton et al., 1994a). This proposal does agree with the most recent X-ray structural model of *Rb. sphaeroides* (Ermler et al., 1994), which furthermore indicates that it is the methoxy group at C_3 which is oriented out of the quinone ring plane while the methoxy at C_2 is oriented parallel to the plane. It is interesting to relate the latter observation to the frequency of the C_1 carbonyl of Q_A, which is found at 1660 cm^{-1}, i.e., at almost the same frequency as the carbonyl proximal to the in-plane methoxy in isolated ubiquinone. These observations further strengthen our previous conclusion on the absence of a hydrogen bond at the C_1 carbonyl of Q_A and on the existence of a very strong hydrogen bond between the $C_4{=}O$ and His M219 downshifting the $C{=}O$ frequency by ~50 cm^{-1} compared to the frequency of the corresponding carbonyl in solution.

Returning to the Q_B site, the absence in the double-difference spectra (Figures 4d,e and 5d,e) of the features characteristic of a $O{-}CH_3$ bond oriented as for the quinone in solution as well as preliminary results obtained on the photoreduction of Q_3 ^{18}O-labeled on both methoxy groups indicating a different spectral behavior in the Q_A or Q_B sites of *Rb. sphaeroides* (J.B., E.N., and C.B., unpublished results) shows that the $O{-}CH_3$ bonds in the Q_B site have a geometry different from that in the Q_A site. This leads us to suggest that the methoxy groups of Q_B are both oriented out of the quinone plane. Such a conformation of higher energy than that of ubiquinone in solution (Burie, 1994) would explain the apparent constraints in the vicinity of the C_4 atom afforded by the protein to anchor the methoxy group at C_3, which are indicated by the present FTIR results. Considering the effect of the geometry of a methoxy group on the frequency of an adjacent $C{=}O$ group, discussed in a previous section for ubiquinone *in vitro*, the proposed out-of-plane conformation of the methoxy groups of Q_B would imply a carbonyl frequency of 1650 cm^{-1} if there was no interaction with the protein. Thus, the 1641-cm^{-1} frequency observed for both carbonyl modes of Q_B appears to favor weak bonding interactions with the protein.[3]

Quantum chemical calculations have shown that the conformation of minimum energy for the semiquinone has both of the $O{-}CH_3$ bonds out of the quinone ring plane (Burie, 1994). It is then tempting to speculate that the two methoxy groups of the neutral Q_B adopt a conformation close to that of the semiquinone in solution. Bringing the neutral Q_B in a conformation close to that of the anion would provide a rationale for the higher potential of Q_B compared to Q_A in *Rb. sphaeroides*. These suggestions, which should only be taken as working hypotheses, are also relevant to the questions of the relative binding affinities of Q_B and Q_B^-, of the role of the 5′-methyl group in the anchoring of these states, of the difference between the protein response to the photoreduction of Q_{10} and Q_0 in the Q_B site (Breton et al., 1991b), and, more generally, of the role of the protein in assisting the electron transfer reaction by bringing the reactants close to the transition state toward the products.

Comparison of the FTIR Data with the X-ray Structures. The clearest result emerging from the present FTIR study concerns the close correspondence of the Q_B vibrations in the RCs of *Rb. sphaeroides* and *Rp. viridis*. Compared to the large variations in the proposed bonding interactions derived from the interpretation of the X-ray data (Michel et al., 1986; Deisenhofer & Michel, 1989; Deisenhofer et al., 1995; Lancaster et al., 1995; Allen et al., 1988; El-Kabbani et al., 1991; Chirino et al., 1994; Ermler et al., 1994), the remarkable equivalence of the various sets of double-difference spectra presented here unambiguously demonstrates the almost identical bonding pattern and conformation of the neutral Q_B in the RCs of the two species. Taking the newest structure of *Rp. viridis* (Lancaster et al., 1995) as the most reliable representation presently available of the Q_B pocket in bacterial RC, it can thus be stated that it also provides the description of the native Q_B site in the RCs of *Rb. sphaeroides*. In our opinion, higher resolution structures should further confirm the close identity of the Q_B conformation in the RCs of both species.

Concerning the conformation of the semiquinone, the similarity in the pattern and the relative amplitudes of the positive and negative bands observed in the various double-difference spectra for the two species also points to a close equivalence of the Q_B^- geometry in the RCs of *Rb. sphaeroides* and *Rp. viridis*. This result becomes even sharper when the double-difference spectra are calculated between the Q_B^-/Q_B spectra obtained for the selective ^{13}C labeling at the 1- or the 4-position (e.g., spectrum 4b minus spectrum 4c). Although in this case the interpretation of the double-difference spectrum is even more complex than for the usual double-difference spectra, this spectrum represents a very specific fingerprint of the effect of moving a single neutron from C_1 to C_4 on the vibrations of Q_B and Q_B^-. In brief, the contribution of the unlabeled and labeled carbonyls of each of the specifically labeled quinones will cancel as they are approximately equivalent in amplitude and frequency and only the differential effects of the selective labeling on the $C{=}C$ vibrations will show up in such a spectrum. When calculated for the RCs of both species, these spectra (Breton et al., 1995) display a remarkable similarity with a positive band at 1616 cm^{-1} flanked by two smaller negative bands at 1626 and 1600 cm^{-1}. In the anion

[3] Such a small frequency downshift could also be caused by an effect of dielectric constant rather than by hydrogen bonding.

absorption region, the similarity is also striking with a large differential signal at 1490/1470 cm^{-1} and a smaller one at 1413/1424 cm^{-1}. Such a striking similarity for the differential effect of selective labeling on the semiquinone C⋯C vibrations was not expected from the direct comparison of the two corresponding sets of double-difference spectra (Figures 4d,e and 5d,e). This appears to be due essentially to the difference in width and peak frequency of the Q_B^- C⋯O mode of the unlabeled Q_3 in the RCs of the two species. As these C⋯O bands essentially cancel each other in the double-difference spectrum for the differential effect of the selective labeling, only the contribution from the C⋯C modes is revealed. The close identity of the fingerprints of the differential effect of the selective labeling on the semiquinone C⋯C modes for the two species provides compelling evidence for a very similar mode of binding of Q_B^- in *Rb. sphaeroides* and *Rp. viridis*. The main remaining difference between the vibrational properties of the semiquinone in the RCs of both species is thus the larger width and the 4-cm^{-1} downshift of the C⋯O mode in *Rp. viridis* compared to *Rb. sphaeroides*. This can be related to the difference in the response of the protein to Q_B^- formation which is clearly detected above 1650 cm^{-1} in the Q_B^-/Q_B FTIR difference spectra (Figures 1a and 2a). Both of these effects are likely to be due to the few residues lining the Q_B pocket that differ between the two organisms, notably, Val L194 in *Rb. sphaeroides* (Ile in *Rp. viridis*), Asp L213 (Asn), Phe L215 (Tyr), and Thr L226 (Ala) although conserved residues in different conformation or environment could also contribute.

Finally, one of the interesting issues addressed by the present work is that of the nature, strength, geometry, and role of the bonding interactions between the protein and the cofactors as they can be proposed from the analysis of the X-ray data or as they are derived from light-induced FTIR spectroscopy. The X-ray structures of the RCs provide information on the direction of a putative hydrogen bond and on the distance between the hydrogen bond donor and acceptor with a mean coordinate error on each of the nonhydrogen atoms which is 0.25−0.3 Å for the best available structures (Lancaster et al., 1995). On the other hand, the frequency of the IR vibrations provides precise information on the perturbation of a given chemical group by the environment. Although, in the long term, these two pieces of information will hopefully converge to give a detailed description of the parameters involved in the binding of the cofactors to the protein, this is not always the case. The reasons for these discrepancies are not yet understood, although a large part of them probably originates from the parameterization of the electron density of the cofactors serving as initial input to analyze the measured electron density map of the crystal as well as from the intrinsic blur in the atomic coordinates. On the other hand, the IR results obtained with the selectively labeled quinones, which, *per se*, do provide the information on the very large difference in the perturbation of the C_1 and C_4 carbonyls, are totally silent on which residue is likely to provide such interactions. Thus, the information arising from both techniques has to be combined.

In the highest resolution X-ray structure of the Q_B site presently available (Lancaster et al., 1995), the carbonyl oxygen at C_1 is proposed to accept two hydrogen bonds from the peptide nitrogens of Ile L224 (at a distance of 3.1 Å) and Gly L225 (distance 3.0 Å) while the C_4 carbonyl accepts a hydrogen bond from the Nδ1 atom of His L190 (distance 2.7 Å), which is also a ligand (at Nε2) to the nonheme iron. This pattern of interactions of His L190 with both the nonheme iron and the C_4=O group of Q_B in *Rp. viridis* is thus very close to that proposed for His M219 with the nonheme iron and the C_4=O group of Q_A in *Rb. sphaeroides* (Ermler et al., 1994) and in *Rp. viridis* (Lancaster et al., 1995). Owing to the pseudo-two-fold symmetry of the RCs, the overall orientation of the hydrogen bond partners is also conserved at the Q_A and Q_B sites. Taken together with the evidence for identical bonding interactions of Q_B in the RC of both species derived from the present FTIR data, the most recent X-ray results (Ermler et al., 1994, Lancaster et al., 1995) suggest that the main difference in the bonding interaction between the C_4 carbonyl of Q_A and Q_B in *Rb. sphaeroides* resides in the shorter hydrogen bond length proposed for Q_B (2.7 Å) than for Q_A (3.2 Å). Thus, the best X-ray structures for the quinone binding sites in RCs give no clues to the large differences in the vibrational frequency of the C_4 carbonyl of Q_A and Q_B detected by light-induced FTIR difference spectroscopy. It is particularly striking that the C_4=O mode of Q_A experiences a much larger downshift (50 cm^{-1}) than that of Q_B (9 cm^{-1}) while, considering the present X-ray structures, one would have made the opposite prediction. These observations also show that electronic coupling of the C_4=O of Q_A with the nonheme iron atom through the imidazole ring of His M219 cannot be the main reason for the strong downshift of the C_4=O of Q_A. A number of these unresolved issues can be addressed by performing further FTIR experiments with selective labeling of either the cofactor or the protein.

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