Binding Sites of Quinones in Photosynthetic Bacterial Reaction Centers Investigated by Light-Induced FTIR Difference Spectroscopy: Binding of Chainless Symmetrical Quinones to the Q_A Site of *Rhodobacter sphaeroides*

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ABSTRACT: Light-induced FTIR Q_A^-/Q_A difference spectra corresponding to the photoreduction of the primary quinone acceptor Q_A have been obtained for *Rhodobacter sphaeroides* RCs reconstituted with chainless symmetrical quinones in order to study the influence of the side chain and of molecular asymmetry on the binding of natural quinones to the Q_A site. The main vibrational modes of the quinones *in vivo* were obtained by analysis of the isotope effects induced by ^{18}O substitution on the carbonyls and by comparison with the IR absorption spectra of the isolated quinones. For isolated 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone (MQ_0), 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone, DQ), and 2,3-dimethyl-1,4-naphthoquinone (DMNQ), the IR spectra together with mass spectroscopy data of partially ^{18}O labeled quinones show that the labeling of one carbonyl leads to only a minor shift of the vibrational frequency of the opposite carbonyl. This observation demonstrates an essentially uncoupled behavior of the two C=O groups. Upon reconstitution of Q_A-depleted RCs with these symmetrical quinones, the double-difference spectra calculated from the Q_A^-/Q_A spectra of the ^{18}O-labeled and unlabeled quinones reveal a splitting of the quinone C=O modes. This splitting and the frequency downshift of the C=O vibrations upon binding to the Q_A site are comparable to those previously reported for the C=O modes of quinones containing an isoprenoid (Q_8, Q_6, Q_1) or a phytyl chain (vitamin K_1) [Breton, J., Burie, J.-R., Berthomieu, C., Berger, G., & Nabedryk, E. (1994) *Biochemistry 33*, 4953–4965]. This observation demonstrates that the replacement of the side chain by a methyl group does not impair the asymmetrical bonding interactions of the two quinone carbonyls with the protein. This asymmetry is traceable to the two distinct amino acid residues which have been proposed, on the basis of X-ray structural studies, to form hydrogen bonds with the carbonyls of the quinone. The close analogy between the double-difference spectra calculated for RCs reconstituted either with vitamin K_1 or with DMNQ shows that the phytyl chain of vitamin K_1 imparts no specific constraint on the geometry of the menaquinone head group in its binding site for both the neutral and the semiquinone state. In contrast, the double-difference spectra calculated for RCs reconstituted either with MQ_0 or with Q_6 (or Q_1) exhibit significant differences in the relative amplitudes of the bands assigned to the mixed C=O and C=C modes of the neutral quinones. This observation shows that, within the first isoprene unit of the side chain, at least one of the carbon atoms beyond the one proximal to the quinone ring must play an important role in allowing the native geometrical anchoring of the ubiquinones to the Q_A site. For all of the investigated quinones, the protein response upon Q_A reduction is highly conserved and the unsplit C=O anion mode indicates symmetrical bonding interactions of the semiquinone with the protein.

In the photosynthetic bacterial reaction center (RC),[1] two quinone molecules (Q_A and Q_B) display very distinct properties: Q_A is a tightly bound one-electron acceptor, while Q_B is loosely bound, can accept two electrons, and serves as a mobile proton carrier (Feher et al., 1989). In the case of *Rhodopseudomonas viridis*, Q_A is a menaquinone and Q_B is a ubiquinone (Q_9), while in *Rhodobacter sphaeroides* both Q_A and Q_B are ubiquinones (both Q_{10}). As a common characteristic, all of these quinones bear a long side chain (9 or 10 isoprene units) which may have a structural role in anchoring the quinone head group to the specific binding sites revealed by X-ray crystallography of the RC protein (Michel et al., 1986; Allen et al., 1988; Deisenhofer & Michel, 1989; El-Kabbani et al., 1991). These quinones are asymmetrical due to the presence of a methyl group adjacent to one carbonyl and of the side chain next to the other carbonyl (Figure 1). In addition, the ubiquinones bear two methoxy groups at the 2- and 3-position of the ring, while menaquinones have an aromatic cycle.

In the past few years, light-induced FTIR difference spectroscopy has emerged as a new tool to investigate the details of the submolecular structural changes occurring at the level of both the quinone itself and the protein upon quinone photoreduction in RCs (Bagley et al., 1990; Bauscher et al., 1993a,b; Breton et al., 1991a,b,c, 1992, 1994; Buchanan et al., 1990, 1992; Hienerwadel et al., 1992; Mäntele et al., 1990; Nabedryk et al., 1990, 1991; Thibodeau et al., 1990a,b). In a recent study, Q_A-depleted *Rb. sphaeroides* RCs were

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[1] Abbreviations: RC, reaction center; P, primary electron donor; Q_A and Q_B, primary and secondary quinone acceptor; *Rb.*, *Rhodobacter*; FTIR, Fourier transform infrared; Q_n, ubiquinone, 2,3-dimethoxy-5-methyl-6-(prenyl)$_n$-1,4-benzoquinone; MQ_0, 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone; DQ, duroquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone; menaquinone, 2-methyl-1,4-naphthoquinone; DMNQ, 2,3-dimethyl-1,4-naphthoquinone; vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone; PQ_9, 2,3-dimethyl-6-nonaprenyl-1,4-benzoquinone.

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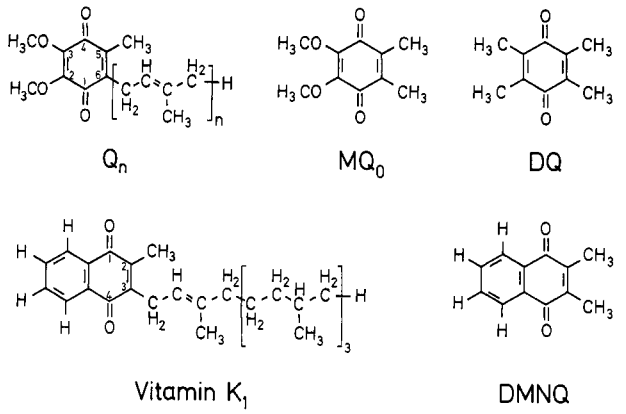

FIGURE 1: Structures of ubiquinone (Q_n), 2,3-dimethoxy-5-methyl-6-(isoprenyl)$_n$-1,4-benzoquinone; MQ_0, 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone; duroquinone (DQ), 2,3,5,6-tetramethyl-1,4-benzoquinone; vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone; and DMNQ, 2,3-dimethyl-1,4-naphthoquinone.

reconstituted either with ubiquinones varying in the length of the isoprenoid chain (Q_1, Q_6, Q_8) or with a menaquinone bearing a phytyl chain (vitamin K_1). Comparison of the Q_A^-/Q_A light-induced FTIR difference spectra obtained with these quinones and with their ^{18}O- or ^{13}C-labeled isotopomers allowed for the first time the modes of Q_A to be discriminated from those of the protein (Breton et al., 1994). Furthermore, the isotopic shifts revealed a considerable splitting of the C=O modes of the neutral quinone for all of the investigated quinones. The large downshift of one C=O mode of Q_A compared to the main frequency of the quinone carbonyls *in vitro* was interpreted in terms of a strong bonding interaction of one C=O group with the protein. The primary origin for this striking asymmetry in the bonding of the two carbonyls to the Q_A site is probably related to the difference in the amino acid residues which have been proposed to participate in the hydrogen bonds to the quinone carbonyls (Michel et al., 1986; Allen et al., 1988; Deisenhofer & Michel, 1989; El-Kabbani et al., 1991; Ermler et al., 1992). However, it is also possible that the presence of the side chain next to one C=O group and of a methyl group next to the other is an important determinant of the strong asymmetry in the bonding of the two quinone carbonyls. One way to discriminate between these two alternatives is to use analogs of the native quinones in which the side chain is replaced by a methyl group, therefore making the quinone a symmetrical molecule with identical substituents next to each carbonyl (Figure 1). In the present work, we report on the Q_A^-/Q_A FTIR spectra of *Rb. sphaeroides* RCs reconstituted with symmetrical analogs of ubiquinones (MQ_0, 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone) and menaquinones (DMNQ, 2,3-dimethyl-1,4-naphthoquinone). The Q_A^-/Q_A spectra and the effect of isotopic substitution on the carbonyl oxygen atoms are compared to that obtained with the more nativelike Q_A molecules bearing a side chain, Q_6 and vitamin K_1, respectively, as well as to those of RCs reconstituted with duroquinone (DQ, 2,3,5,6-tetramethyl-1,4-benzoquinone), a quinone with the highest possible level of symmetry.

MATERIALS AND METHODS

RCs from *Rb. sphaeroides* (strain R26) were purified according to the method of Clayton and Wang (1971), and the native Q_{10} was extracted from the Q_A and Q_B sites according to Okamura et al. (1975), as modified by Woodbury et al. (1986). Control measurements have shown that more than 98% of Q_A has been extracted (Breton et al., 1994). The RCs were stored at a concentration of ≈0.5 mM at −70 °C

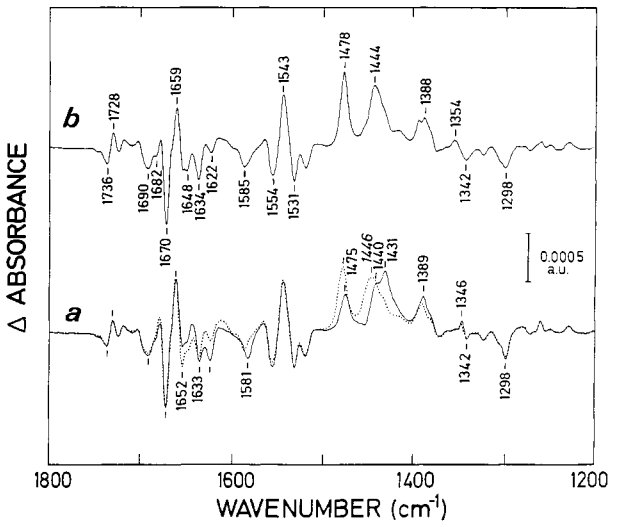

FIGURE 2: Light-induced Q_A^-/Q_A FTIR difference spectra of Q_A-depleted *Rb. sphaeroides* RCs reconstituted (a) with unlabeled DMNQ (···) or ^{18}O-labeled DMNQ (—) and (b) with unlabeled vitamin K_1; 5 °C; 4-cm^{-1} resolution. The frequencies of the IR bands are given with an accuracy of +1 cm^{-1}; a.u., absorbance units.

in 100 mM Tris-HCl buffer (pH 7.0) containing 0.1% of the detergent lauryldimethylamine *N*-oxide. Samples for the FTIR measurements were reconstituted with the quinones dissolved in *n*-hexane, as described previously (Breton et al., 1994). The mediator used for rapid rereduction of the oxidized primary electron donor was *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The Q_A to Q_B electron transfer was blocked by 20 mM *o*-phenanthroline.

Vitamin K_1, Q_6, and DQ (all from Sigma), DMNQ (Aldrich), and MQ_0 (Apin Chemicals Ltd., U.K.) were labeled and purified following procedures previously described (Berger et al., 1994; Breton et al., 1994). Mass spectroscopy was performed upon electron impact on a Finnigan 4600 instrument.

Light-induced FTIR measurements were performed under steady-state illumination on a Nicolet 60SX spectrometer as previously described (Breton et al., 1994). The spectra obtained with the ^{18}O-labeled quinones were corrected for the incomplete labeling of the quinones according to the average ^{18}O incorporation values obtained by mass spectroscopy.

RESULTS

Q_A^-/Q_A FTIR Difference Spectra. The light-minus-dark Q_A^-/Q_A FTIR difference spectrum of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with DMNQ [Figure 2a, dotted line, and Breton et al. (1992)] is remarkably close to that obtained when the RCs are reconstituted with vitamin K_1 [Figure 2b and Breton et al. (1992, 1994)]. Apart from minor changes in the relative amplitude of a number of bands, the main differences are changes in the shape of the negative band at ≈1650 cm^{-1}, of the positive band at ≈1390 cm^{-1}, and of the differential signal at ≈1350 cm^{-1} as well as the 2-cm^{-1} shift of the anion band at 1444 cm^{-1}.[2] When the RCs are reconstituted with ^{18}O-labeled DMNQ, additional changes are observed (Figure 2a, solid line) which closely resemble those previously reported for ^{18}O-labeled vitamin K_1 (Breton et al., 1994). These changes are localized in the region 1660–1570 cm^{-1}, where the C=O and C=C bands of the isolated

[2] In a Q_A^-/Q_A spectrum, the bands of the neutral quinone are expected to show a negative sign, while the bands of the semiquinone should appear positive. Additional contributions 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_A are also present in the difference spectra.

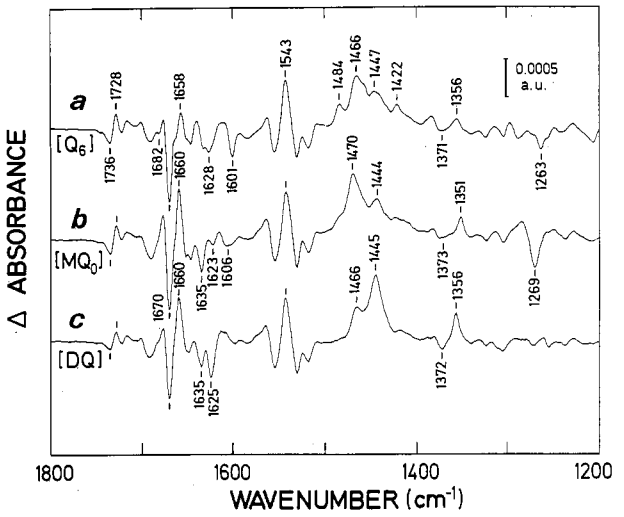

FIGURE 3: Q_A^-/Q_A spectra of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with unlabeled quinones: (a) Q_6, (b) MQ_0, and (c) DQ.

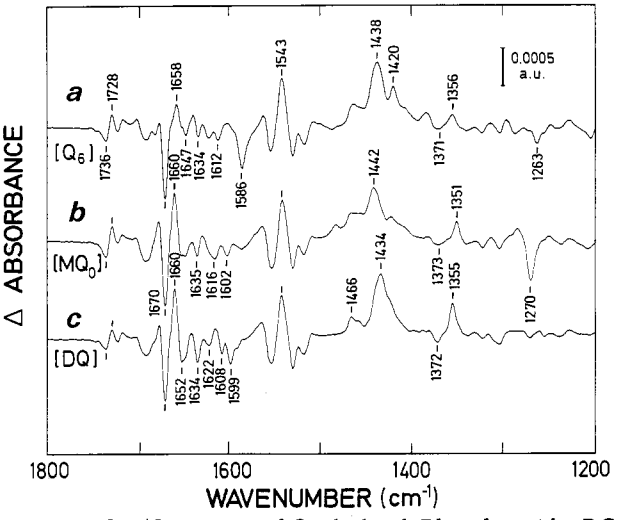

FIGURE 4: Q_A^-/Q_A spectra of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with quinones ^{18}O-labeled on their carbonyls: (a) Q_6, (b) MQ_0, and (c) DQ.

neutral quinones are found (Breton et al., 1992, 1994), and between 1500 and 1370 cm^{-1}, where the C⋯O and C⋯C bands of the semiquinones absorb *in vitro* (Breton et al., 1991b; Bauscher & Mäntele, 1992). Upon ^{18}O substitution, the 1478-cm^{-1} band decreases in amplitude and downshifts to 1475 cm^{-1}, a large fraction of the band at 1446 cm^{-1} downshifts by 15 cm^{-1}, and the band at 1389 cm^{-1} increases slightly. In a previous study of RCs reconstituted with ^{18}O-labeled vitamin K_1, a possible contribution to the anion band around 1440 cm^{-1} of vibrations of the phytyl chain, which absorbs in this frequency range, could not be ruled out (Breton et al., 1994). This possibility can now be excluded, as the 1440-cm^{-1} mode is still present in the Q_A^-/Q_A spectrum of RCs reconstituted with ^{18}O-labeled DMNQ (Figure 2a, solid line). Thus, the 1440-cm^{-1} anion band of DMNQ and vitamin K_1 is more probably related to an aromatic C⋯C mode.

As reported previously, the Q_A^-/Q_A spectrum of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with Q_6 (Figure 3a), Q_8, or Q_{10} is indistinguishable from that of RCs containing the native Q_{10} (Breton et al., 1991a, 1992, 1994). It is also very close to that of RCs reconstituted with Q_1 (Breton et al., 1994). In contrast, these spectra are distinctly different from that recorded with RCs reconstituted with MQ_0 (Figure 3b). Compared to the Q_A^-/Q_A spectrum of Q_6, amplitude increase of bands at 1660, 1470, and 1351 cm^{-1} (positive) and at 1635 cm^{-1} (negative) can be noticed as well as the decrease of the amplitude of bands of Q_6 at 1484 cm^{-1} (positive) and at 1628 and 1601 cm^{-1} (negative). The negative band at 1263 cm^{-1} (Figure 3a), previously assigned to C–O–CH_3 vibrations from the methoxy groups of Q_6 (Breton et al., 1994), appears to gain intensity and to shift to 1269 cm^{-1} (Figure 3b).

The Q_A^-/Q_A spectrum of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with DQ (Figure 3c) also exhibits large differences from that of RCs reconstituted with Q_6 (Figure 3a), notably the development of a negative band at 1625 cm^{-1}, the replacement of the structured positive feature peaking at 1466 cm^{-1} by a main band at 1445 cm^{-1} with a satellite peak at 1466 cm^{-1}, and a large increase of the positive peak at 1356 cm^{-1}. Several of these features can also be recognized in a previously reported Q_A^-/Q_A spectrum of *Rb. sphaeroides* RCs reconstituted with DQ (Bauscher et al., 1993b). In the Q_A^-/Q_A spectra obtained with the chainless quinones (Figure 3b,c), additional differences are also observed in the 1750–1650-cm^{-1} frequency range. The most noticeable ones are a change in the amplitude of the positive band at 1660 cm^{-1}, which is more developed than in the Q_A^-/Q_A spectrum of Q_6, and a decrease of the small negative band at 1682 cm^{-1}. In the case of RCs reconstituted with DQ, the amplitude of the differential signal at 1728/1736 cm^{-1} appears reduced relative to the amplitude of the other bands.

The main isotope effects in the Q_A^-/Q_A spectra of the RCs reconstituted with ^{18}O-labeled Q_6, MQ_0, and DQ (Figure 4) are found in the range 1670–1580 cm^{-1} where the C=C and C=O vibrations of the neutral quinones *in vitro* are found (Bagley et al., 1990; Bauscher & Mäntele, 1992; Breton et al., 1994) and in the 1500–1400-cm^{-1} range of absorption of the C⋯O and C⋯C modes of the semiquinones *in vitro* (Bauscher et al., 1990; Bauscher & Mäntele, 1992). For RCs reconstituted with Q_6 (Figures 3a and 4a), these changes have already been described and interpreted (Breton et al., 1994). In the case of RCs reconstituted with MQ_0, the band at 1635 cm^{-1} decreases in amplitude upon labeling (Figure 4b) and new bands appear at 1616 and 1602 cm^{-1}. In the semiquinone absorption region, the main band at 1470 cm^{-1} decreases strongly while the band at 1444 cm^{-1} increases and downshifts slightly. The Q_A band at 1269 cm^{-1} appears almost unaffected by the labeling. For RCs reconstituted with DQ, the amplitude of the band at 1625 cm^{-1} decreases upon ^{18}O substitution while two negative bands develop at 1608 and 1599 cm^{-1} (Figure 4c). In the semiquinone absorption region, the band at 1466 cm^{-1} decreases and the main band at 1445 cm^{-1} downshifts to 1434 cm^{-1}. The 1356-cm^{-1} band is almost unaffected by the ^{18}O substitution.

Spectra of the Isolated Quinones. The absorption bands of the neutral isolated unlabeled DMNQ (Figure 5a) are very similar to those of vitamin K_1 (Breton et al., 1992, 1994), except for the absence of a band at 1461 cm^{-1} which in vitamin K_1 corresponds to δCH_2 and δCH_3 vibrations from the phytyl chain (Bellamy, 1980; Breton et al., 1994). The band at 1377 cm^{-1} in the absorption spectrum of DMNQ (Figure 5a), which is absent in the spectrum of unsubstituted 1,4-naphthoquinone and exhibits a reduced amplitude in the spectrum of 2-methyl-1,4-naphthoquinone (Breton et al., 1992), can be assigned to the δCH_3 mode (Bellamy, 1980) from the methyl groups attached to the quinone ring (Figure 1). Similarly to vitamin K_1 (Breton et al., 1994), the bands at 1662, 1624, and 1598 cm^{-1} of DMNQ (Figure 5a) are assigned to the C=O, the quinonic C=C, and the aromatic C=C mode, respectively. Only the middle band is significantly upshifted (6 cm^{-1}) compared to the corresponding mode of vitamin K_1, demonstrating the presence of a coupling between the quinonic

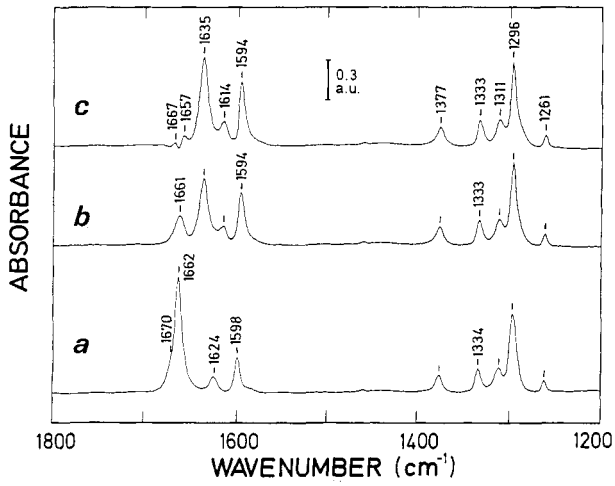

FIGURE 5: IR absorption spectra of (a) unlabeled DMNQ and (b) ^{18}O-labeled DMNQ (experimental). Solvent (CCl_4) absorption was subtracted, and spectra were normalized on the bands below 1400 cm^{-1}. (c) IR absorption spectrum of ^{18}O-labeled DMNQ (calculated). This spectrum takes into account the average ^{18}O labeling (73%) determined from mass spectroscopy analysis.

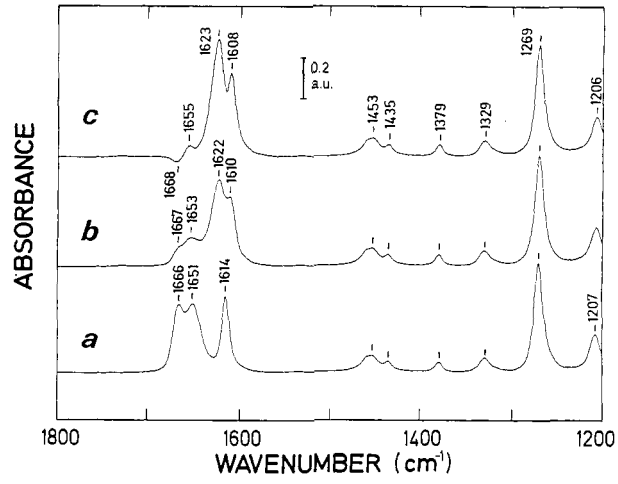

FIGURE 6: IR absorption spectra of (a) unlabeled $MQ_{0\ and}$ (b) ^{18}O-labeled MQ_0 (experimental). Solvent (CCl_4) absorption was subtracted, and spectra were normalized on the bands below 1500 cm^{-1}. (c) IR absorption spectrum of ^{18}O-labeled MQ_0 (calculated). This spectrum takes into account the average ^{18}O labeling (75%) determined from mass spectroscopy analysis.

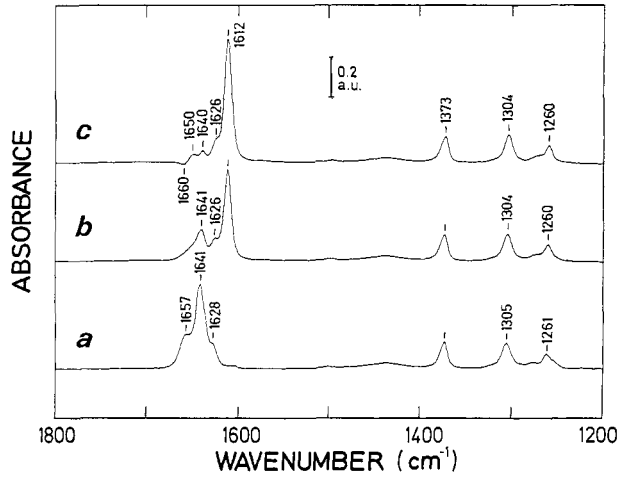

FIGURE 7: IR absorption spectra of (a) unlabeled DQ and (b) ^{18}O-labeled DQ (experimental). Solvent (CCl_4) absorption was subtracted, and spectra were normalized on the bands below 1400 cm^{-1}. (c) IR absorption spectrum of ^{18}O-labeled DQ (calculated). This spectrum takes into account the average ^{18}O labeling (72%) determined from mass spectroscopy analysis.

C=C mode and the side chain in vitamin K_1. Upon ^{18}O labeling, new bands appear at 1635, 1614, and 1594 cm^{-1} (Figure 5b). The conditions used for the labeling, and notably the use of unlabeled TFA, lead to a partial incorporation of ^{18}O in the labeled quinone, as reflected by the band remaining at 1661 cm^{-1} (Figure 5b). It should be noted that this band has an amplitude of about 25% of that of the unlabeled DMNQ relative to the bands below 1400 cm^{-1} and that its frequency is very little affected by the labeling, being downshifted by only 1 cm^{-1} compared to its peak position for the unlabeled quinone. Analysis of the purified quinones by mass spectroscopy reveals that 54% of the DMNQ molecules bear ^{18}O on both of their carbonyls, and 38% bear ^{18}O on only one carbonyl, while 8% remain unlabeled. This pattern confirms that, as expected, the isotopic exchange is a random process rather than a concerted one in which both oxygen atoms would be simultaneously exchanged. From the fraction of unlabeled quinone (8%), it can be deduced that the band remaining at 1661 cm^{-1} in the experimental spectrum of the ^{18}O-labeled DMNQ (Figure 5b) is for a large part contributed by the ^{16}O C=O stretching vibration of DMNQ molecules that have incorporated a single ^{18}O label. An average ^{18}O labeling of 73% is calculated from the sum of the population (54%) of doubly labeled DMNQ and of one-half of the population (38%) of singly labeled quinones. After normalization of the two experimental spectra (Figure 5a,b) on the bands below 1400 cm^{-1}, which appear essentially unaffected by the ^{18}O labeling, a corrected ^{18}O DMNQ spectrum can be obtained (Figure 5c) by subtracting 27% of the spectrum of unlabeled DMNQ (Figure 5a) from the experimental spectrum of the ^{18}O-labeled quinone (Figure 5b). The factor applied to the experimental spectra to generate this corrected spectrum takes into account only the net ^{18}O incorporation and does not distinguish between the singly (^{16}O ^{18}O) and the doubly (^{18}O ^{18}O) labeled quinone. The small residual bands observed around 1660 cm^{-1} in the corrected spectrum of the ^{18}O-labeled DMNQ (Figure 5c) are taken to reflect the slight frequency shift of the unlabeled C=O mode of DMNQ in response to the labeling of the other carbonyl (see Discussion).

The absorption spectrum of the isolated MQ_0 (Figure 6a) shows two carbonyl bands of equal amplitude at 1666 and 1651 cm^{-1} and a large C=C band at 1614 cm^{-1}. By comparison with the absorption spectra of DQ (Figure 7a) as well as of Q_1, Q_0, and 2,3-dimethoxy-1,4-benzoquinone (not shown), the bands at 1453 and 1435 cm^{-1} are assigned to vibrations from the methyls of the methoxy groups, in agreement with Bellamy (1980). The band at 1379 cm^{-1} is assigned to the δCH_3 mode of the methyl groups directly attached to the quinone ring. The 15-cm^{-1} downshift of the corresponding band observed upon uniform ^{13}C labeling of Q_8 (Breton et al., 1994) is indicative of some coupling of this asymmetric CH_3 bending mode with deformation of the C_5–CH_3 bond. The band at 1269 cm^{-1} appears to correspond to the 1263-cm^{-1} band of Q_6 attributed to the C–O–C vibrations of the methoxy groups (Bellamy, 1980). Upon ^{18}O labeling and normalization of the spectra (Figure 6a,b) on the bands below 1500 cm^{-1}, a corrected MQ_0 spectrum (Figure 6c) can be calculated, taking into account the average incorporation of the ^{18}O label (75%) determined by mass spectroscopy. After correction, the C=C band shifts from 1614 to 1608 cm^{-1} upon ^{18}O labeling, while the two C=O bands appear to merge in a rather broad band peaking at 1623 cm^{-1} (Figure 6c). The small bands around 1660 cm^{-1} are thought to arise from a small frequency shift of the ^{16}O C=O vibration of MQ_0 molecules upon incorporation of an ^{18}O isotope on the other carbonyl.

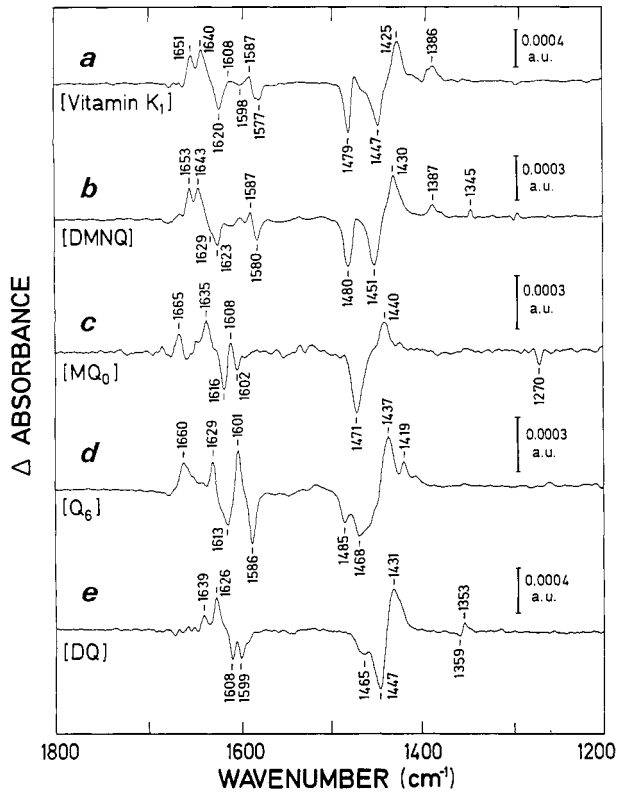

FIGURE 8: Double-difference spectra (^{18}O minus ^{16}O) obtained from pairs of Q_A^-/Q_A spectra of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with either ^{18}O-labeled or unlabeled quinones. For each pair of spectra, the Q_A^-/Q_A spectrum obtained with RCs reconstituted with unlabeled quinone was subtracted from that obtained with RCs reconstituted with ^{18}O-labeled quinone. (a) Vitamin K_1, (b) DMNQ, (c) MQ_0, (d) Q_6, and (e) DQ.

The absorption spectrum of the isolated DQ (Figure 7a) exhibits a main band at 1641 cm^{-1} that shifts to 1612 cm^{-1} upon ^{18}O labeling (Figure 7b,c) and is thus assigned to the main C=O mode. A smaller band at about 1657 cm^{-1}, which appears to vanish upon ^{18}O substitution, probably corresponds to a splitting of the C=O modes due to Fermi resonance (see Discussion). The band at ≈1628 cm^{-1} seems little affected by the ^{18}O substitution and is thus likely to originate from the C=C mode. As reported above for DMNQ and MQ_0, the small bands around 1650 cm^{-1} in the spectrum (Figure 7c) of the ^{18}O-labeled DQ corrected for the presence of the residual unlabeled compound according to the mass spectroscopy results (72% average incorporation) are attributed to the shift in frequency of the ^{16}O C=O vibration of DQ molecules bearing an ^{18}O isotope on the opposite carbonyl. The band at 1373 cm^{-1} is assigned to the δCH_3 vibration of the methyl groups attached to the ring. Interestingly, a negative band at almost the same frequency (1372 cm^{-1}) is seen in the Q_A^-/Q_A spectrum of RCs reconstituted with DQ (Figure 3c). This band is unaffected by ^{18}O labeling both *in vitro* and *in vivo*.

Double-Difference Spectra. Contributions from the quinone vibrations themselves *in vivo* can be separated from those of the protein by calculating the double-difference spectrum between the pair of Q_A^-/Q_A spectra recorded with the isotopically labeled and unlabeled quinones (Breton et al., 1994). Such a double-difference spectrum (^{18}O-labeled minus unlabeled) for DMNQ (Figure 8b) is compared to the double-difference spectrum previously reported for Q_A-depleted *Rb. sphaeroides* RCs reconstituted with unlabeled and ^{18}O-labeled vitamin K_1 [Figure 8a and Breton et al. (1994)]. In these double-difference spectra, the bands of the neutral unlabeled quinone *in vivo* are expected to appear with a positive sign, while those of the neutral labeled quinone should contribute with a negative sign. The opposite situation applies to the bands of the semiquinones. The double-difference spectra for DMNQ (Figure 8b) and for vitamin K_1 (Figure 8a) are remarkably similar in shape, and the frequencies of most of the bands also agree quite closely. The major differences are a ≈3-cm^{-1} upshift of the main bands of the neutral DMNQ (at 1653, 1643, and 1623 cm^{-1}) compared to the frequencies of the equivalent bands of vitamin K_1 (at 1651, 1640, and 1620 cm^{-1}) and a ≈4-cm^{-1} upshift of the 1451/1430-cm^{-1} differential signal originating from the shift of the 1446-cm^{-1} anion band. The frequencies of the positive and negative bands in the 1670–1570-cm^{-1} frequency range of the double-difference spectrum (Figure 8b) correspond well to the main C=O and C=C absorption bands of the isolated unlabeled and ^{18}O-labeled DMNQ molecules (Figure 5a,c), respectively, although a downshift is observed for the *in vivo* vibrations (Table 1).

Table 1: Frequencies (cm^{-1}) of Carbonyl IR Bands of Menaquinones (Vitamin K_1, DMNQ) and Benzoquinones (Q_6, MQ_0, DQ) *in Vitro* and in *Rb. sphaeroides* RCs[a]

	in vitro	in *Rb. sphaeroides* RCs	δ
vitamin K_1	1661	1651	10
		1640	21
DMNQ	1662	1653	9
		1643	19
Q_6	1664	1660	4
	1650	1628	22
MQ_0	1666	1665	1
	1651	1635	16
DQ	1641	1639	2
		1626	15

[a] δ, observed frequency downshift (cm^{-1}) upon binding to the RC.

In contrast to the very comparable double-difference spectra obtained for DMNQ and vitamin K_1, the double-difference spectrum derived from Q_A^-/Q_A spectra of RCs reconstituted with MQ_0 (Figure 8c) is quite different from that of RCs reconstituted with Q_6 [Figure 8d and Breton et al. (1994)]. Although the set of bands reproducibly observed at 1665, 1635, and 1608 cm^{-1} (positive) and at 1616 and 1602 cm^{-1} (negative) in the double-difference spectrum for MQ_0 resemble those at 1660, 1629, and 1601 cm^{-1} (positive) and at 1613 and 1586 cm^{-1} (negative) in the double-difference spectrum for Q_6, the relative amplitudes and the frequencies of the bands differ significantly. The bands located above 1580 cm^{-1} in the double-difference spectrum for MQ_0 (Figure 8c) and for Q_6 (Figure 8d) are within the range of absorption of the C=O and C=C vibrations of the neutral quinones. The close correspondence of the frequencies of these bands for MQ_0 (Figure 6a,c) and for Q_6 *in vitro* (Breton et al., 1994; see also Table 1) together with the significant changes observed in the shapes of the double-difference spectra for these two ubiquinones (Figure 8c,d) indicate that a definite change in the protein–quinone interactions occurs upon replacement of the isoprenoid chain of Q_6 by a methyl group. In the region of absorption of the C=O and C=C bands of the semiquinone, the changes occurring upon ^{18}O labeling in the Q_A^-/Q_A spectrum of RCs reconstituted with MQ_0 lead to the rather simple differential signal negative at 1471 cm^{-1} and positive at 1440 cm^{-1} in the double-difference spectrum (Figure 8c), which is less structured than the one obtained for Q_6 (Figure 8d).

When the double-difference spectrum for the RCs reconstituted with DQ (Figure 8e) is compared to the absorption spectra of the isolated DQ molecules (Figure 7a,c), the doublets of positive (^{16}O) bands at 1639 and 1626 cm^{-1} and of negative (^{18}O) bands at 1608 and 1599 cm^{-1} *in vivo* appear downshifted

relative to the main C=O band at 1641 (^{16}O) and 1612 cm^{-1} (^{18}O) in the *in vitro* spectra (Figure 7a,c and Table 1). The high-frequency band in each doublet is only slightly downshifted (by 2–4 cm^{-1}), while the lower frequency one is much more affected (15–13 cm^{-1}). In the region of the C···O and C···C bands of the semiquinone, the absorption decrease at 1466 cm^{-1} and the 11-cm^{-1} downshift of the main anion band at 1445 cm^{-1} observed in the Q_A^-/Q_A spectra upon ^{18}O labeling of DQ lead to the structured 1447/1431-cm^{-1} differential signal in the double-difference spectrum (Figure 8e). The small derivative signal at 1359/1353 cm^{-1} can be related to a small (≈1 cm^{-1}) but highly reproducible downshift of the 1356-cm^{-1} positive band in the Q_A^-/Q_A spectra upon labeling (Figures 3c and 4c).

DISCUSSION

Effect of Isotopic Substitution on the Quinone Bands in Vitro. In addition to the isotope effects on the frequency of the vibrational bands, which have already been discussed and utilized in previous work (Breton et al., 1994) for the band assignment of vitamin K_1 and of various ubiquinones (Q_6, Q_8) *in vitro*, two new observations deserve discussion. First, the MQ_0 molecule also exhibits a splitting (≈15 cm^{-1}) of the carbonyl bands as previously observed for Q_6 (Breton et al., 1994) as well as for Q_0 and decyl Q_0 (data not shown). This splitting is generally assigned to a differential effect of the distinct substituents at the 5- and 6-positions on the frequency of the two C=O modes (Meyerson, 1985). In view of the symmetry of the MQ_0 molecule, this interpretation does not hold, and the splitting of the carbonyl bands has to be assigned either to Fermi resonance or to a difference in the geometry of the two methoxy groups. The former effect corresponds to a coupling between two degenerate levels, e.g., a C=O vibration and the overtone of a mode at half the frequency of the C=O vibration (Becker et al., 1963). A splitting caused by Fermi resonance can sometimes be ascertained by isotope substitution because the frequency shift of the C=O uncouples the mixing and leads to the appearance of a single band as, e.g., in 1,4-benzoquinone (Becker et al., 1963). Although the absence of a clear splitting of the C=O bands in the absorption spectrum of ^{18}O-labeled MQ_0 (Figure 6c) suggests that this interpretation is to be favored, additional information coming both from normal mode analysis and from selective ^{13}C labeling at the 1- or 4-position of the ring in Q_3 (to be published) indicates that the splitting is rather due to a different conformation of the two methoxy groups.

Second, the small signals observed between 1670 and 1640 cm^{-1} in the absorption spectra of the isolated ^{18}O-labeled quinones corrected for the incomplete labeling (Figures 5c, 6c, and 7c) are also worth discussion. In a symmetric quinone, the two C=O groups are expected to perform coupled vibrations (Girlando & Pecile, 1979), and a similar coupling could still be maintained in the case of asymmetrically substituted quinones. However, the influence of this coupling on the frequency of the IR modes has, to our knowledge, not yet been estimated. For a strong coupling effect, the frequency of the C=O mode of that population of the quinones bearing a ^{18}O atom on one carbonyl and a ^{16}O atom on the other should lie close to the average between the C=O frequencies of the unlabeled and the fully labeled quinone. This is clearly not observed experimentally (Figures 5–7). The frequency of an ^{16}O carbonyl of DMNQ bearing an ^{18}O label on the opposite C=O can be obtained upon correction of the experimental spectra of the ^{18}O quinone (Figure 5b) for the amount of unlabeled compound (8%) determined from the mass spectroscopy analysis. The resulting spectrum (not shown) only contains the contributions of the (^{16}O ^{18}O) and (^{18}O ^{18}O) quinone populations. For DMNQ, the frequency of the ^{16}O carbonyl band ascribed to the (^{16}O ^{18}O) population is found at 1660 cm^{-1}, compared to 1662 cm^{-1} for the unlabeled compound. Thus, the shift in frequency of an ^{16}O carbonyl upon replacement of the second carbonyl oxygen by ^{18}O is only ≈2 cm^{-1} for DMNQ. Similarly, a downshift of ≈1 cm^{-1} is found for the 1641-cm^{-1} band of DQ, while an upshift of ≈2 cm^{-1} is apparent for the 1651-cm^{-1} band of MQ_0 with no clear evidence of a shift for the 1666-cm^{-1} carbonyl band. These small shifts of the ^{16}O C=O mode upon ^{18}O labeling of the opposite carbonyl are not specific to symmetrical quinones, as the same behavior has also been observed upon partial ^{18}O labeling of Q_6 and vitamin K_1 (not shown). The presence of high-energy shoulders in the absorption spectrum of unlabeled DMNQ (Figure 5a) and DQ (Figure 7a), most likely related either to Fermi resonance or to the normally IR-inactive symmetric C=O mode, complicates a quantitative analysis of the influence of the coupling on the frequency of the C=O modes. A comparable complication is found for MQ_0 with the splitting of the C=O modes assigned to a different geometry of the two methoxy groups. Nevertheless, the present result provides the first evidence that coupling of the vibrations of the C=O groups has only minor spectroscopic consequences on the frequency of the carbonyl bands of benzoquinones and menaquinones. Notably, this allows the two C=O bands of ubiquinones to be essentially considered as the vibrational modes of each individual C=O group. In addition, this property of the two carbonyls of quinones to behave spectroscopically like almost isolated oscillators allows frequency shifts as a function of an external perturbation, e.g., hydrogen bonding, to be calculated for each of the two C=O groups.

C=O and C=C Bands of the Neutral Quinones in Rb. sphaeroides. Using *Rb. sphaeroides* RCs reconstituted with isotopically labeled quinones containing an isoprenoid (Q_1, Q_6, Q_8) or a phytyl chain (vitamin K_1), it has been shown that the two carbonyls of Q_A are engaged in highly asymmetrical bonding interactions with the protein at the binding site. Moreover, the isotopic shifts revealed that the C=C mode and the strongly downshifted C=O mode both have a highly mixed character (Breton et al., 1994).

Similarly to the case of vitamin K_1, the doublet of positive bands at 1653 and 1643 cm^{-1} in the double-difference spectrum of DMNQ *in vivo* (Figure 8b) is interpreted as the C=O modes of the two differently bound carbonyls of the unlabeled DMNQ species.[3] Compared to the peak position at 1662 cm^{-1} for the isolated quinone, the downshifts are 9 and 19 cm^{-1} for the quinone *in vivo* (Table 1). These shifts are close to those (10 and 21 cm^{-1}, respectively) reported for vitamin K_1 in the Q_A site [Breton et al. (1994) and Table 1]. The broad negative band at 1623 cm^{-1} with a shoulder at 1629 cm^{-1} (Figure 8b) is assigned to the overlap of the two C=O modes of the ^{18}O-labeled DMNQ that exhibit nonidentical shifts due to their different coupling to the C=C modes as previously described for vitamin K_1 (Breton et al., 1994). The negative band at 1580 cm^{-1} is assigned to the aromatic C=C mode of the ^{18}O-labeled DMNQ, which is downshifted by 14 cm^{-1} compared to its position *in vitro* (1594 cm^{-1}). While the isotopic shift of this mode is only 4 cm^{-1} for the isolated molecule, it is about 7 cm^{-1} for DMNQ in the Q_A site. As previously discussed for vitamin K_1, this is taken to indicate a larger

[3] On the basis of a comparison of the Q_A^-/Q_A spectra of *Rb. sphaeroides* RCs reconstituted with a series of substituted 1,4-naphthoquinones, one of the C=O modes of DMNQ in the Q_A site had been previously proposed to absorb at 1643 cm^{-1} (Breton et al., 1992).

mixing of the C=O and C=C modes *in vivo* than *in vitro*. The close analogy between the double-difference spectra calculated for RCs reconstituted either with vitamin K_1 [Breton et al. (1994) and Figure 8a] or with DMNQ (Figure 8b) provides strong evidence that the phytyl chain of vitamin K_1 imparts no specific constraint on the anchoring of the menaquinone head group in its binding site for both the neutral and the semiquinone state of Q_A.

For MQ_0 in the Q_A site, the double-difference spectrum upon ^{18}O substitution (Figure 8c) reveals a splitting of the two C=O bands of unlabeled MQ_0 *in vivo* by 30 cm^{-1}, which is almost identical to that (31 cm^{-1}) observed for Q_6 *in vivo* [Breton et al. (1994) and Figure 8d] while the splitting of the two C=O bands for both MQ_0 and Q_6 *in vitro* is only about half that value (Table 1). The large splitting of the two C=O bands *in vivo* is assigned to the different bonding interactions of the two carbonyl groups of these quinones in the Q_A site. The significant differences observed in the frequency and relative amplitude of the bands assigned to the vibrations with mixed C=O and C=C character in the 1630–1580-cm^{-1} frequency range of the double-difference spectra for MQ_0 and Q_6 are taken to reflect a change in the geometry of binding of these two quinones *in vivo* (see following discussion).

For DQ in the Q_A site of *Rb. sphaeroides*, the double-difference spectrum (Figure 8e) also reveals two positive bands at 1639 and 1626 cm^{-1} and two negative bands at 1608 and 1599 cm^{-1}. Although it cannot be excluded that the binding of DQ to the Q_A site could alter the mixing of the C=O and C=C characters of the IR vibrations, the location of the main C=O band of DQ *in vitro* and the isotopic shift upon ^{18}O labeling rather lead us to assign these bands in the double-difference spectrum to the two differently downshifted C=O modes *in vivo*. The band at 1626 cm^{-1} closely corresponds to a band at 1625 cm^{-1} in the $P^+Q_A^-/PQ_A$ spectrum of *Rb. sphaeroides* RCs reconstituted with DQ which had been previously assigned to the C=O mode of DQ (Bagley et al., 1990). The isotopic shifts of 31 and 27 cm^{-1} observed for the bands of the two carbonyls of DQ *in vivo* upon ^{18}O substitution are very close to the corresponding shift (29 cm^{-1}) found in the isolated DQ molecule. This near identity of the ^{18}O isotopic shifts of both carbonyl bands *in vivo*, which had never been observed with all the previously examined quinones reconstituted in RCs, is probably related to the high symmetry of DQ leading to a coupling of the C=O and C=C modes that is different from that present in less symmetrical quinones.

The observation that the coupling of the vibrations of the two C=O groups has little effect on the frequency of the carbonyl bands *in vitro* suggests that the C=O groups can also be viewed as independent oscillators *in vivo*. If this is the case, it follows that the frequency of the carbonyl bands of Q_A can be correlated to the amount of perturbation experienced by each of the C=O groups. The good agreement found between the binding free energy of the quinones to the protein at the Q_A site, as measured from binding constant studies (Gunner et al., 1985, 1986; Woodbury et al., 1986; Gunner, 1991; Warncke & Dutton, 1993) and as estimated from the shift of the frequency of the C=O bands upon binding (Breton et al., 1994), provides support for this hypothesis. The *in vivo* and *in vitro* frequencies of the main carbonyl bands of the various quinones investigated in the present study are listed in Table 1. For unlabeled DMNQ and vitamin K_1, the two neutral carbonyls *in vivo* are both downshifted compared to the position of the carbonyl band *in vitro*, with one of them having been shifted approximately twice as much as the other. In contrast, only one of the carbonyl bands of the three benzoquinones used for reconstitution (Q_6, MQ_0, and DQ) is strongly downshifted *in vivo* compared to its position *in vitro*, while the other one is only very slightly shifted. Considering the C=O groups of these quinones *in vivo* as isolated oscillators, the frequency of the carbonyl bands (Table 1) indicates that the binding of the benzoquinones to the protein at the Q_A site occurs essentially by only one carbonyl, while that of the menaquinones is less asymmetrical. This difference in the binding asymmetry of the carbonyls to the protein, with essentially one anchoring point for the benzoquinones and two anchoring points for the menaquinones, might explain the different behavior observed in the present study upon replacement of the side chain by a methyl group for the two classes of quinones.

In the RC of photosystem II, both the primary and the secondary acceptors are plastoquinones (PQ_9). A common characteristic of DQ and PQ_9 is the presence of methyl groups at the 2- and 3-position of the benzoquinone ring where ubiquinones have methoxy groups. Two bands at 1645 and 1630 cm^{-1} in the Q_A^-/Q_A spectrum of photosystem II have been tentatively assigned to the carbonyl modes of PQ_9 *in vivo* (Berthomieu et al., 1990, 1992). These two bands are downshifted by 6 and 21 cm^{-1} with respect to the main C=O band of PQ_9 *in vitro* (1652 cm^{-1}). In *Rb. sphaeroides* RCs reconstituted with Q_6, the observed downshifts of the carbonyls upon binding are 4 and 22 cm^{-1} (Breton et al., 1994). The similarity of the shifts of the frequency of the carbonyls upon binding has been taken as evidence that these two evolutionarily related RCs (Michel & Deisenhofer, 1988) exhibit a similar bonding pattern of the primary quinone with conserved amino acid residues (Breton et al., 1994). However, it could not be excluded that one of the main chemical differences between Q_6 and PQ_9, namely, the replacement of the two methoxy groups present in Q_6 by methyl groups in PQ_9, would invalidate this comparison. In *Rb. sphaeroides* RCs reconstituted with DQ, the corresponding downshifts of the carbonyls upon binding are 2 and 15 cm^{-1}. These shifts are almost identical to those (1 and 16 cm^{-1}) observed for MQ_0 (Table 1). Thus, the main determinant of the splitting of the C=O modes of the quinones in the Q_A site of *Rb. sphaeroides* and of photosystem II appears related more to the asymmetry of the bonding interactions of the carbonyls with the protein than to an effect of the change between methoxy and methyl substituents.

C⋯O and C⋯C Bands of the Semiquinones in Rb. sphaeroides In the region of absorption of the C⋯O and C⋯C vibrations of the semiquinones *in vitro* (1500–1400 cm^{-1}), the overall structures of the Q_A^-/Q_A spectra of RCs reconstituted with the three benzoquinones described here exhibit some similarities, notably with a set of overlapping bands (Figures 3 and 4). In contrast, they are quite different from those of DMNQ and vitamin K_1, which are characterized by three well-separated bands (Figure 2). In the Q_A^-/Q_A spectra of RCs reconstituted with Q_6, MQ_0, and DQ, the main anion band peaks at 1466, 1470, and 1445 cm^{-1}, respectively. Upon ^{18}O substitution on the quinone carbonyls, this main anion band downshifts by 28 cm^{-1} for both Q_6 and MQ_0 and by 11 cm^{-1} for DQ. For a pure C=O stretching mode at this frequency, the calculated shift gives 35 cm^{-1}. The observed shifts are within the range of those expected for a C⋯O vibration coupled to C⋯C modes and are thus consistent with the assignment of the main anion band to predominantly C⋯O modes proposed previously for Q_6 (Breton et al., 1994). Furthermore, the 1451/1430-cm^{-1} differential signal in the double-difference spectrum for DMNQ *in vivo* (Figure 8b) shows a ≈4-cm^{-1} upshift compared to the equivalent feature at 1447/1425 cm^{-1} of vitamin K_1 (Figure 8a), while the other

anion bands in the double-difference spectrum exhibit only a $\approx$1-cm^{-1} upshift. This shift can be related to the $\approx$3-cm^{-1} upshift of the *in vivo* frequency of the C=O bands of the neutral DMNQ compared to vitamin K_1, while the aromatic C=C mode is found at the same frequency (1587 cm^{-1}) for the two quinones *in vivo*. The comparison of these shifts is taken to further support the C$\therefore$O character previously assigned to the 1444-cm^{-1} anion band of vitamin K_1 (Breton et al., 1994). The absence of a splitting of the main anion band for all the investigated quinones strengthens the notion of symmetrical bonding interactions of the two C$\therefore$O groups of the semiquinone with the protein proposed previously (Breton et al., 1994). In the electrochemically generated anion spectra of model quinones *in vitro* (Bauscher et al., 1990; Breton et al., 1991b; Bauscher & Mäntele, 1992), the C$\therefore$O mode is upshifted by 20–50 cm^{-1} compared to the frequency observed for the corresponding mode *in vivo* (Figures 2 and 3). This observation agrees with the notion of strong hydrogen bonds between the protein and the two carbonyls of the semiquinone derived from ENDOR studies of Q_A^- (Feher et al., 1985).

The anion band at $\approx$1484 cm^{-1} is well defined in the Q_A^-/Q_A spectrum of RCs reconstituted with Q_6 (Figure 3a) or Q_1 (Breton et al., 1994). It is present as a shoulder in the corresponding Q_A^-/Q_A spectra recorded with either MQ_0 (Figure 3b) or decyl Q_0 (not shown) but is absent in the spectra of DQ (Figure 3c). When present, this band exhibits variable sensitivity to ^{18}O substitution on the quinone carbonyls: it disappears in Q_6, decreases in Q_1 (Breton et al., 1994), and is unaffected in MQ_0 (Figures 3b, 4b, and 8c). This band, which appears sensitive both to the nature of the substituent at the 6-position of the ring and to the labeling of the quinone carbonyls, is thus tentatively assigned to C$\therefore$C vibrations involving both the quinone ring and the C_6–C_{chain} mode (or the C_{ring}–CH_3 mode in MQ_0).

Other Vibrations of the Quinones in Rb. sphaeroides. In the Q_A^-/Q_A spectrum of RCs reconstituted with DQ (Figure 3c), a strong positive band at 1356 cm^{-1} can be correlated with a negative band at 1372 cm^{-1} which corresponds closely to a band at 1373 cm^{-1} in the absorption spectrum of the isolated DQ (Figure 7a). This band is currently assigned to δCH_3 vibration of the four methyl groups (Bellamy, 1980). The *in vitro* anion-minus-neutral spectrum of DQ in various solvents (Bauscher, 1991) exhibits a differential signal closely corresponding in shape and frequency to the 1356/1372-cm^{-1} differential signal of the Q_A^-/Q_A spectrum of RCs reconstituted with DQ (Figure 3c). A comparable differential signal, although of reduced amplitude, is also present in the Q_A^-/Q_A spectrum of RCs reconstituted either with MQ_0 (Figure 3b) at 1351/1373 cm^{-1}, with Q_6 (Figure 3a) at 1356/1371 cm^{-1}, or with Q_1 (Breton et al., 1994) at 1356/1370 cm^{-1}. The amplitude of these differential signals *in vivo* roughly correlates with the number of methyl groups directly attached to the benzoquinone ring. These differential signals in the Q_A^-/Q_A spectra, which are only very little affected by the ^{18}O isotopic substitution on the quinone carbonyls (Figures 3 and 4), are assigned to the effect of quinone photoreduction on the δCH_3 vibration of the methyl group(s) attached to the benzoquinone ring. The frequencies of this vibration for the quinone in solution and in the protein binding site are quite close. This is taken to indicate that these methyl groups are not much influenced by the binding of the quinone to the Q_A site. However, the larger downshift of this vibration upon binding to the protein observed for Q_6 ($\approx$10 cm^{-1}) and MQ_0 (6 cm^{-1}) than for DQ (1 cm^{-1}) suggests either an influence of the number of methyl substituents on the amplitude of this downshift or some specificity of the interaction between the methyl groups of these different quinones and the protein. Ubiquinones selectively labeled at the 5-position of the ring and/or on the methyl group as well as normal mode calculations will be needed to further quantify the degree of deformation of this substituent upon binding.

The intense negative band at 1269–1270 cm^{-1} in the Q_A^-/Q_A spectrum of RCs reconstituted with MQ_0 (Figures 3b and 4b), which corresponds to a band of large amplitude at the same frequency in the absorption spectrum of isolated MQ_0 (Figure 6), is assigned to C–O–C vibrations from the methoxy groups. This band is the equivalent of the 1263-cm^{-1} band of Q_6 both *in vitro* and *in vivo*. As previously discussed for the Q_A^-/Q_A spectra of Q_6 *in vivo*, the methoxy groups of MQ_0 appear essentially unaffected by the binding to the protein.

Protein Response to Q_A Photoreduction. Besides the vibrations of the quinone itself, which can be ascertained by reconstituting the RCs with chemical analogs and with isotopically labeled compounds, the Q_A^-/Q_A spectra contain many contributions from non-quinone vibrations. These non-quinone contributions are found not only in spectral regions where quinones do not contribute, e.g., above 1670 cm^{-1} or in the 1570–1500-cm^{-1} frequency range but also in the 1670- to 1570-cm^{-1} region where the quinone C=O and C=C vibrations absorb. Although the nature of the substituents attached to a 5,6-dimethyl-1,4-benzoquinone head group has been extensively varied in the present study (two methoxy groups in MQ_0, two methyl groups in DQ, or one aromatic cycle in DMNQ), one striking observation is that the protein signals appear generally much less affected by these changes of the substituents than the quinone vibrations themselves. This conclusion is best illustrated by comparing the Q_A^-/Q_A spectra obtained with the three corresponding quinones (Figures 2a and 3b,c). The largest variations between these spectra appear related to bands that show isotope effects upon labeling of the quinone carbonyls, while only little variation is observed in the regions outside the range of absorption of the quinones. This observation indicates that upon Q_A photoreduction the protein is responding essentially to the effect of the charge on the quinone ring while possible effects at the level of the substituents remain undetected. Furthermore, the close equivalence of the Q_A^-/Q_A spectra for DMNQ and vitamin K_1 as well as for Q_1 and Q_6 demonstrates that the IR vibrations corresponding to interactions of the protein with the quinone side chain remain essentially unperturbed upon Q_A photoreduction.

While most of the non-quinonic signals in the Q_A^-/Q_A spectra are insensitive to the chemical nature of the quinone head group acting in the Q_A site, the amplitude of a few bands appears to depend on the presence or absence of a side chain on the quinone. This is notably the case for a small negative band present at 1682 cm^{-1} in the Q_A^-/Q_A spectra of RCs reconstituted with vitamin K_1 or with Q_6, which is reduced to a small shoulder in all the spectra of RCs reconstituted with chainless quinones. Similarly, the amplitude of the positive band at 1660 cm^{-1} is generally larger for the chainless quinones than for either vitamin K_1 or Q_6. The amplitude of the 1728/1736-cm^{-1} derivative signal relative to that of the 1670-cm^{-1} band is smaller for DMNQ and DQ compared to the case of vitamin K_1 or Q_6. Whether these effects are solely due to the replacement of the side chain by a methyl group or to some more subtle perturbation of the quinone–RC complex cannot be ascertained from the present data.

Influence of the Side Chain on the Quinone Vibrations. The definite splitting exhibited by the C=O modes of prenylated or phytylated quinones naturally present or introduced into the Q_A site of *Rb. sphaeroides* RCs (Breton et al., 1994) could, in principle, be explained by two non-

exclusive mechanisms. On the one hand, the presence of the side chain next to one carbonyl and of a methyl group next to the other imparts an asymmetry to the quinone that is sufficient to allow a specific differential binding of these two substituents by the protein. Under such conditions, each of the two carbonyls may be forced into very different interactions with the protein. On the other hand, it is also possible that the difference between the methyl and side chain substituents has little influence on the inequivalent binding of the quinone carbonyls and that the main reason for the splitting of the C=O modes is to be found in the different natures of the amino acid residues which have been proposed to participate in the hydrogen bonds to the quinone carbonyls (Michel et al., 1986; Allen et al., 1988; Deisenhofer & Michel, 1989; El-Kabbani et al., 1991; Ermler et al., 1992). From the present work it is concluded that the splitting of the C=O modes is maintained when these quinones are replaced by symmetrical analogs in which the side chain has been changed to a methyl group. Thus, the main determinant of the asymmetry of the carbonyls of quinones bearing a side chain in the Q_A site of *Rb. sphaeroides* is not the difference between a chain or a methyl group as substituent but rather the intrinsic inequivalence of the bonding interactions with the protein. In principle, the present results obtained with the symmetrical quinone analogs do not allow us to exclude that the asymmetry exhibited by the carbonyls *in vivo* is due to a strong differential anchoring to the protein of only one of the two methyl substituents. However, the absence of splitting of the negative band at $\approx$1372 cm^{-1} and of the associated downshifted anion peak characteristic for these methyl groups makes this interpretation quite unlikely. The asymmetry of the carbonyls *in vivo* is thus more probably caused by the two distinct residues which have been proposed to form hydrogen bonds with the carbonyls of the quinone, i.e., Ala M260 on the carbonyl proximal to the side chain and either His M219 or Thr M222 on the carbonyl proximal to the methyl group.

Analysis of the Q_A^-/Q_A spectra of *Rb. sphaeroides* RCs reconstituted with isotopically labeled quinones has revealed that the binding of Q_6 or of vitamin K_1 to the Q_A site has a very specific influence on the C=O and C=C vibrations of these two quinones (Breton et al., 1994). The comparison of the Q_A^-/Q_A spectra of RCs containing either vitamin K_1 (Figure 2b) or an analog in which the side chain at the 3-position is replaced by a methyl group (Figure 2a) and the analysis of the isotope effects observed upon ^{18}O substitution (Figure 8a,b) demonstrate that the absence of the side chain does not perturb the binding of these menaquinones to the Q_A site. In contrast, the replacement of the side chain by a methyl group brings significant perturbations to the binding of ubiquinones to the Q_A site (see following discussion). It can thus be surmised that the aromatic cycle in DMNQ provides a more rigid anchoring of the quinone head group to the Q_A site than that afforded by the methoxy groups of MQ_0. In this respect the presence of the planar aromatic cycle of menaquinones seems to favor the formation of the two distinct bonding interactions of the carbonyls with the protein, while the more flexible methoxy groups of ubiquinones would lead to only one strong bond.

The double-difference spectrum for MQ_0 in the Q_A site shows some significant differences when compared to the equivalent spectrum for Q_6 in the Q_A site, notably in the relative amplitudes of the C=O and C=C bands of the neutral quinones. The large differential feature of Q_6 at 1601/1586 cm^{-1} (Figure 8d) is considerably affected in the case of MQ_0 and is replaced by the small 1608/1602-cm^{-1} signal (Figure 8c). On the basis of a comparison of the frequency of the IR bands of Q_6 observed *in vivo* and of the C=O and C=C modes of the isolated molecule, the bands at 1628 and 1601 cm^{-1} of unlabeled Q_6 in the Q_A site have been provisionally assigned to predominantly C=O and C=C modes, respectively. From the observed isotope effects, however, it is clear that both bands have a highly mixed C=O and C=C character (Breton et al., 1994).[4] The present data demonstrate that the replacement of the side chain in Q_6 by a methyl group in MQ_0 modifies the coupling of the C=O and C=C modes *in vivo* and suggest that this replacement is sufficient to lead to a definite change in the geometry of the ubiquinone head group in the Q_A site. The closeness of the Q_A^-/Q_A spectra obtained with native RCs and with RCs reconstituted either with Q_6 or with Q_1 and the similar isotope effects observed on these spectra (Breton et al., 1994) have been taken to demonstrate that a possible role of the side chain in providing the native geometry of the ubiquinone head group in the Q_A protein binding site for both the neutral and the semiquinone state could not extend beyond the first isoprene unit (Breton et al., 1994). This observation concurs with the report that a specific region within the first two isoprene units has an important influence on the binding affinity of ubiquinones to the Q_A site of *Rb. sphaeroides* RCs (Warnke et al., 1987). Furthermore, while the change of binding affinity for the series of chain-bearing ubiquinones (Q_1 to Q_{10}) is quite independent of the number of isoprene units, the removal of the last unit in Q_0 has a marked influence on the binding at the Q_A site (McComb et al., 1990). The large difference between the amplitude and frequency of the C=O and C=C quinone vibrations in the Q_A^-/Q_A spectra of MQ_0 on the one hand and those of Q_1 or Q_6 on the other hand further demonstrates that an important determinant of the native anchoring of the ubiquinone head group in the Q_A binding domain is contained within the five carbon atoms of the first isoprene unit of the side chain. Notably, a specific role of one or more of the last four carbon atoms of the first isoprene unit is revealed in the present study. Using FTIR difference spectroscopy of Q_A photoreduction, it would thus be of interest to assess ubiquinones with short alkyl or unsaturated chains at the 6-position of the quinone ring to further precisely define the influence of the various C—C or C=C bonds of the side chain on the interactions of the quinone with the protein.

Just before submission of this work, we became aware of the recent results of Warncke et al. (1994) on the influence of the hydrocarbon side chain structure on the binding free energies of ubiquinones and menaquinones at the Q_A site. For short *n*-alkyl chains, it was found that each carbon atom of the side chain has a specific positive or negative contribution to the binding affinity. For ubiquinones, the first and fourth carbon atoms of the side chain make a favorable contribution to the affinity, while the second, the third, and the fifth make unfavorable contributions. This pattern of domains of alternating favorable and unfavorable contributions along the side chain has been further traced down to cavities and to regions of steric hindrance in the protein regions of the X-ray structures that accommodate the methyl and methylene groups of the side chain. The precise docking of these groups into their respective protein domains would help secure the quinone head group into the Q_A site. The present FTIR data show that the strong bonding interaction with the protein of one carbonyl of MQ_0 in the Q_A site is not sufficient to achieve the same native geometry of the head group as found for Q_1. It is thus likely that the specific set of differential affinities of the first few carbons of the side chain in their binding domains together with the strong bonding of one of the quinone

[4] See Added in Proof.

carbonyls are essential determinants in providing the native geometrical anchoring of the ubiquinone head group to the Q_A site. For menaquinones, the influence of the first carbon atoms of the side chain on the binding affinity to the Q_A site was found to be much less pronounced than for ubiquinones bearing a side chain of identical structure (Warncke et al., 1994). This is consistent with our finding of an identical geometry of the menaquinone head group for DMNQ and for vitamin K_1 in the Q_A site.

ADDED IN PROOF

Recently, Q_A^-/Q_A FTIR spectra have been obtained for *Rb. sphaeroides* RCs reconstituted with ubiquinone (Q_3) labeled selectively with ^{13}C at the 1- or 4-position of the quinone ring, i.e., on either one of the two carbonyls (J.B., C.B., J.-R.B., E.N., and C. Mioskowski, submitted to *Biochemistry*). Double-difference spectra calculated from the Q_A^-/Q_A spectra of the $^{13}C_1$- or $^{13}C_4$-labeled and the unlabeled Q_3 reveal the frequency of the C=C and C=O bands associated with the two carbonyls *in vivo*. The 1660-cm^{-1} band of Q_A is unaffected upon selective labeling at C_4 and downshifts to 1623 cm^{-1} upon $^{13}C_1$ labeling, demonstrating that this band arises from the C_1=O carbonyl, proximal to the isoprenoid chain. The band at 1628 cm^{-1} that donwshifts by 11 and 16 cm^{-1} upon $^{13}C_1$ and $^{13}C_4$ labeling, respectively, is assigned to a C=C mode coupled to both carbonyls. The band at 1601 cm^{-1}, which downshifts to 1578 cm^{-1} upon labeling at C_4 and is unaffected by labeling at C_1, corresponds to the C_4=O carbonyl, proximal to the methyl group. This new assignment scheme differs from the provisional one proposed previously (Breton et al., 1994) by exchanging simply the predominance of the C=O or C=C character of the 1628- and 1601-cm^{-1} modes. However, these new data do not alter the conclusions of the present work regarding the influence of the chain on the different geometries of binding of Q_6 and MQ_0 to the Q_A site of *Rb. sphaeroides*.

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