Binding Sites of Quinones in Photosynthetic Bacterial Reaction Centers Investigated by Light-Induced FTIR Difference Spectroscopy: Assignment of the Interactions of Each Carbonyl of Q_A in *Rhodobacter sphaeroides* Using Site-Specific ^{13}C-Labeled Ubiquinone

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ABSTRACT: Light-induced Q_A^-/Q_A FTIR difference spectra of the photoreduction of the primary quinone (Q_A) have been obtained for *Rhodobacter sphaeroides* reaction centers (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 of the two carbonyls. The vibrational modes of the quinone in the Q_A site are compared to those *in vitro*. IR absorption spectra of films of the labeled quinones show that the two carbonyls contribute equally to the split C=O band at 1663−1650 cm^{-1}. This splitting is assigned to the two different geometries of the methoxy group nearest to each carbonyl. The Q_A^-/Q_A spectra of RCs reconstituted with either $^{13}C_1$- or $^{13}C_4$-labeled Q_3 and with unlabeled Q_3 as well as the double differences calculated from these spectra exhibit distinct isotopic shifts for the bands assigned to C=O and C=C vibrations of the neutral Q_A. For the unlabeled Q_A, these bands correspond to the bands at 1660, 1628, and 1601 cm^{-1} previously detected upon nonselective isotopic labeling [Breton, J., Burie, J.-R., Berthomieu, C., Berger, G., & Nabedryk, E. (1994) *Biochemistry 33*, 4953−4965]. The 1660-cm^{-1} band is unaffected upon selective labeling at C_4 but shifts to ~1623 cm^{-1} upon $^{13}C_1$ labeling, demonstrating that this band arises from the C_1 carbonyl, proximal to the isoprenoid chain. The band at 1628 cm^{-1} shifts by 11 and 16 cm^{-1} upon $^{13}C_1$ and $^{13}C_4$ labeling, respectively, and is assigned to a C=C mode coupled to both carbonyls. The band at 1601 cm^{-1}, which shifts to 1578 cm^{-1} upon labeling at C_4 and is unaffected by labeling at C_1, corresponds to the C_4 carbonyl, proximal to the methyl group. Additional ^{18}O labeling on the carbonyls of the selectively labeled Q_3 confirms these assignments. The large difference in the IR frequencies of the two C=O modes of Q_A underscores the inequivalent interactions of the two carbonyls with the protein. The extreme downshift of the frequency of the C_4=O group in the Q_A binding site compared to that *in vitro*, together with the strongly mixed C=C and C=O characters of the 1628- and 1601-cm^{-1} modes, points to a strong perturbation of the C_4 carbonyl. The large downshift of C_4=O probably is caused by hydrogen bonding with the imidazole ring of His M219, which is located close to this carbonyl group in the most recent X-ray structure of the RC [Ermler, U., Fritzsch, G., Buchanan, S., & Michel, H. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. I, pp 341−347, Kluwer Academic Publishers, Dordrecht]. This hydrogen bond would be stabilized by the linkage of Nϵ2 of the imidazole to the non-heme Fe^{2+}. However, the FTIR data do not support the suggestion based on the X-ray structure that the C_1 carbonyl forms a hydrogen bond with the peptide NH of Ala M260; C_1=O appears not to interact significantly with the protein. In contrast to the uncoupled behavior of the C=O modes of the neutral Q_A, the two C···O modes of the semiquinone in the Q_A^-/Q_A spectra are coupled. They are also coupled to the C···C modes and are both strongly downshifted compared to the C···O mode of the semiquinone *in vitro*. Double-difference spectra calculated from the $P^+Q_A^-/PQ_A$ spectra at 100 K are very similar to the Q_A^-/Q_A double-difference spectra at 278 K, showing that the center of mass of Q_A does not move appreciably upon reduction.

The crystal structure of the photosynthetic bacterial reaction center (RC)[1] suggests that the interactions between the protein and the cofactors involved in electron transport are important both for the geometrical organization of the electron-transfer pathway and for fine tuning the energy levels of its individual components. For example, the very distinct roles in electron and proton transport of the primary (Q_A) and secondary (Q_B) quinones, which in *Rhodopseudomonas viridis* are menaquinone-9 and ubiquinone-9 (Q_9), respectively, and in *Rhodobacter sphaeroides* are both Q_{10}, appear traceable to differences in the nature and packing of the amino acid residues in their respective binding sites [for a review, see Feher et al. (1989)]. Among the essential quinone−protein interactions, hydrogen bonds between the carbonyls of the quinones and proton-donating groups of the

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

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protein have been proposed from binding affinity studies (Gunner et al., 1985, 1986; Warncke & Dutton, 1993; Warncke et al., 1994), from ENDOR spectroscopy (Feher et al., 1985), and from the X-ray structure of RCs from *Rb. sphaeroides* (Allen et al., 1988; El-Kabbani et al., 1991; Ermler et al., 1992) and *Rp. viridis* (Michel et al., 1986; Deisenhofer & Michel, 1989). However, the present state of the analysis of the X-ray data on *Rb. sphaeroides* and *Rp. viridis* RCs leaves several ambiguities regarding these bonding interactions of both Q_A and Q_B with the protein. In particular, while the present X-ray structures of the RC of *Rp. viridis* (Deisenhofer & Michel, 1989) and *Rb. sphaeroides* (Allen et al., 1988; El-Kabbani et al., 1991; Ermler et al., 1992) propose a conserved hydrogen bond between the Q_A carbonyl that is nearest to the isoprenoid chain and the peptide NH of Ala M258 and Ala M260, respectively, they differ with regard to the hydrogen bond partner to the quinone C=O group nearest to the methyl substituent. The most recently described *Rb. sphaeroides* structure (Ermler et al., 1992) proposes that this partner is the His M219 residue analogous to His M217 that participates in the hydrogen bond in *Rp. viridis*. In the two other structures of *Rb. sphaeroides*, the hydrogen bond to this C=O group is to the OH side chain of Thr M222 (Allen et al., 1988; El-Kabbani et al., 1991). In addition, the relative strength of the bonding interactions with each of the two carbonyls is not clearly established, although the shortest proposed distance is toward the peptide NH of Ala. Pending the availability of higher resolution X-ray structures, the determination of the nature and relative strength of the bonding interactions of Q_A with the protein must rely 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 Q_A with the protein, light-induced FTIR difference spectroscopy appears well suited to investigate both the neutral and the 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,b; Buchanan et al., 1990, 1992; Mäntele et al., 1990; Nabedryk et al., 1990, 1991; Thibodeau et al., 1990a,b). Using Q_A-depleted *Rb. sphaeroides* RCs reconstituted with isotopically labeled ubiquinones, the C=O and C=C vibrational modes of Q_A could be determined (Breton et al., 1994a). One band at 1660 cm^{-1} was assigned to an essentially free carbonyl. Two other bands at 1628 and 1601 cm^{-1} exhibit a highly mixed C=O and C=C character. By comparison with the absorption spectra of the isolated quinones, it was tentatively proposed that the 1601-cm^{-1} band corresponds to the C=C mode, while the 1628-cm^{-1} band represents a bound carbonyl group. 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 in the bonding interactions of the two carbonyls of Q_A is not caused by the difference in the substituents at the 5- and the 6-position of the ubiquinone (Figure 1, inset) but rather by the different proteic environment of the two carbonyls (Breton et al., 1994b). Although the conclusion that the Q_A carbonyls form only one strong hydrogen bond compares well with that previously obtained from the binding studies, it contrasts with the interpretation of recent magic angle spinning NMR results on selectively ^{13}C-labeled Q_{10} in the Q_A site of *Rb. sphaeroides* (van Liemt et al., 1993; van Liemt, 1994). In the NMR study, it was concluded that, although the two carbonyls of Q_A are inequivalent, neither is involved in a strong hydrogen bond. Thus, the issue of the bonding interaction of the carbonyls of Q_A in *Rb. sphaeroides* RCs is far from being settled. Furthermore, in the previous FTIR studies, the effect of isotopic labeling on both of the carbonyls was analyzed, and thus the question of which of the two quinone carbonyls is involved in the strong bonding interaction with the protein could not be addressed. This question, which requires the use of *site-specific isotope labeling* of one or the other of the carbonyls, has been investigated in the present FTIR study for ubiquinone in the Q_A site of *Rb. sphaeroides*.

MATERIALS AND METHODS

Ubiquinone (Q_3) selectively labeled with ^{13}C at the 1- or the 4-position was synthesized by the procedure of Rüttimann and Lorenz (1990) from specific ^{13}C-labeled methylsuccinic anhydrides (C. Boullais and C. Mioskowski, unpublished results). Controls by mass spectrometry have shown a ^{13}C incorporation larger than 99%. Unlabeled Q_3 was prepared by using the same procedure. Recently, the synthesis of ^{13}C-labeled Q_{10} by the same route has been reported (van Liemt et al., 1994).

Q_A-depleted RC samples from *Rb. sphaeroides* R-26 were reconstituted with unlabeled and ^{13}C-labeled Q_3 as previously described (Breton et al., 1994a). Incubation of the reconstituted RCs in $H_2{}^{18}O$ for 2–3 days leads to ^{18}O labeling of the two quinone carbonyls (Breton et al., 1994a). Light-induced IR and near-IR measurements were performed under steady-state illumination at 5 °C or at 100 K as reported in Breton et al. (1994a) and Nabedryk et al. (1990). The Q_A^-/Q_A spectra have been corrected for the small contribution from $TMPD^+/TMPD$ (Breton et al., 1992).

RESULTS

Absorption Spectra of the Isolated Quinones. The absorption spectra of films of unlabeled, $^{13}C_1$-labeled, and $^{13}C_4$-labeled Q_3 presented in Figure 1 have been normalized on the bands at 1451 and 1437 cm^{-1} originating from δCH_2 and δCH_3 vibrations of the chain and δCH_3 modes of the methoxy groups (Bellamy, 1980), which appear essentially unaffected by the isotope labeling. Both of the C=O bands at 1663 and 1650 cm^{-1} decrease by about 50% upon labeling, and new bands appear at 1618–1620 and 1601 cm^{-1}, while a shoulder remains at 1611 cm^{-1}. Similar effects have also been reported on the IR spectra of Q_{10} selectively labeled with ^{13}C at the same positions (van Liemt, 1994).

Q_A^-/Q_A FTIR Difference Spectra. The Q_A^-/Q_A spectrum of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with unlabeled Q_3 (Figure 2a) is essentially indistinguishable from that of native RCs and of RCs reconstituted with Q_6 or Q_8 (Breton et al., 1994a). In these spectra, the bands of the neutral Q_A state appear as negative signals, while the positive bands belong to the Q_A^- state. When the RCs are reconstituted with selectively ^{13}C-labeled Q_3, the positions of several bands in the Q_A^-/Q_A spectra are significantly different from those found with unlabeled Q_3 and depend upon

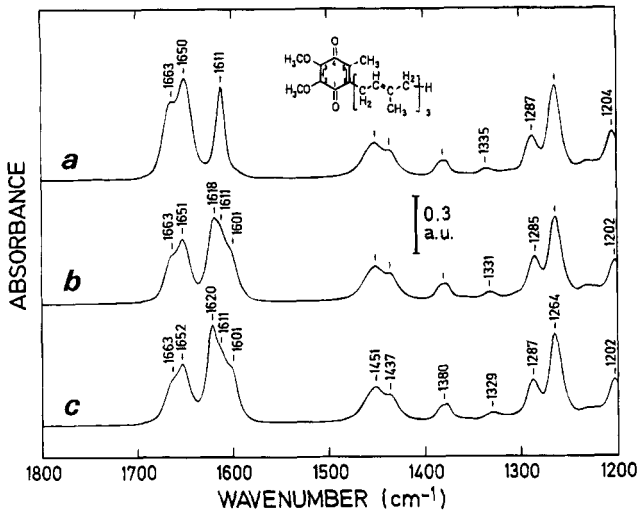

FIGURE 1: IR absorption spectra of films of (a) unlabeled Q_3, (b) $^{13}C_1$-labeled Q_3, and (c) $^{13}C_4$-labeled Q_3. The individual spectra were normalized as described in the text. Inset: Structural formula of ubiquinone-3 (Q_3: 2,3-dimethoxy-5-methyl-6-triprenyl-1,4-benzoquinone).

whether the label is at the 1- (Figure 2b) or the 4-position (Figure 2c). Isotope effects of comparable magnitude have been reported previously upon uniform ^{13}C labeling of Q_8 or ^{18}O labeling of both carbonyls of Q_6. These differences occur in the spectral range 1530−1665 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 1628 cm^{-1} in the Q_A^-/Q_A spectrum of unlabeled Q_3 (Figure 2a) disappears upon site-specific ^{13}C labeling (Figure 2b,c). New negative bands are seen at 1619 cm^{-1} upon $^{13}C_1$ labeling (Figure 2b) and at 1611 and 1578 cm^{-1} upon $^{13}C_4$ labeling (Figure 2c). While the 1601-cm^{-1} band is unaffected by the $^{13}C_1$ labeling, it disappears upon $^{13}C_4$ labeling. Conversely, an increase of the positive band at 1658 cm^{-1} is observed for $^{13}C_1$ labeling but not for $^{13}C_4$ labeling. Large isotope effects are also observed in the semiquinone absorption region, with notably pronounced amplitude changes of the 1484-cm^{-1} band. New bands appear at 1442 and 1419 cm^{-1} for $^{13}C_1$ labeling and at 1430 and 1417 cm^{-1} for $^{13}C_4$ labeling.

$P^+Q_A^-/PQ_A$ FTIR Difference Spectra at 100 K. The $P^+Q_A^-/PQ_A$ spectra at 100 K of RCs reconstituted with unlabeled Q_3 and with either $^{13}C_1$- or $^{13}C_4$-labeled Q_3 (Figure 3a−c) show only small isotope effects, as previously reported for RCs containing isotopically labeled Q_{10} (Bagley et al., 1990). These effects are mainly limited to the regions between 1415 and 1455 cm^{-1} and between 1590 and 1630 cm^{-1}, notably with the disappearance of the small negative band at 1604 cm^{-1} in the spectra of the $^{13}C_4$-labeled Q_3.

Double-Difference Spectra. Isotope-sensitive vibrations from the quinone itself in the Q_A^-/Q_A spectra can be separated from those of the protein by calculating the double-difference spectrum between a pair of Q_A^-/Q_A spectra recorded with RCs reconstituted with isotopically labeled and unlabeled quinones (Breton et al., 1994a,b). Such double-difference spectra are shown for $^{13}C_1$ (Figure 2d) and $^{13}C_4$ labeling (Figure 2e). In these double-difference (isotopically labeled minus unlabeled) spectra, the IR bands of the neutral unlabeled Q_A appear with a positive sign, while the down-

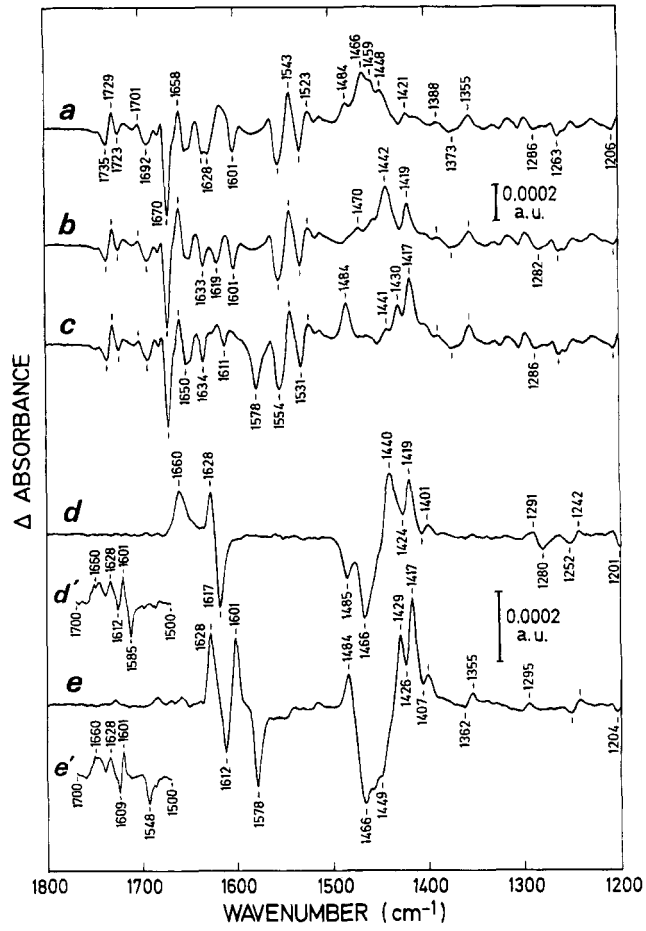

FIGURE 2: Light-induced Q_A^-/Q_A FTIR difference spectra at 5 °C of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with (a) unlabeled Q_3, (b) $^{13}C_1$-labeled Q_3, and (c) $^{13}C_4$-labeled Q_3 and double-difference spectra (isotopically labeled minus unlabeled) obtained for $^{13}C_1$-labeled Q_3 (d), $^{13}C_1$,^{18}O-labeled Q_3 (d'), $^{13}C_4$-labeled Q_3 (e), and $^{13}C_4$,^{18}O-labeled Q_3 (e'); average of 2−4 different pairs of samples. For each pair of spectra, the Q_A^-/Q_A spectrum obtained with RCs reconstituted with unlabeled Q_3 was subtracted from that obtained with RCs reconstituted with isotopically labeled Q_3. For d' and e', the scales have been reduced by one-half. a. u., absorbance units; ~100 000 interferograms added; 4-cm^{-1} resolution. The frequencies of the IR bands are given with an accuracy of ±1 cm^{-1}.

shifted 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. 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, as, e.g., for the negative band corresponding to the downshifted 1660-cm^{-1} band in Figure 2d.

The C=O and C=C vibrations of the neutral unlabeled Q_A lead to the three positive bands at 1660, 1628, and 1601 cm^{-1} in the double-difference spectra of the nonspecifically labeled quinones (Breton et al., 1994a). In the case of the selectively labeled Q_3, only two of these three positive bands are seen (Figure 2d,e). Upon $^{13}C_4$ labeling, the 1660-cm^{-1} band is missing (Figure 2e), showing that this Q_A vibration is unaffected by the labeling at the 4-position. The two positive bands at 1628 and 1601 cm^{-1} are downshifted to 1612 and 1578 cm^{-1}, respectively. Upon $^{13}C_1$ labeling, it is the band at 1601 cm^{-1} that disappears in the double-difference spectrum (Figure 2d), demonstrating that this

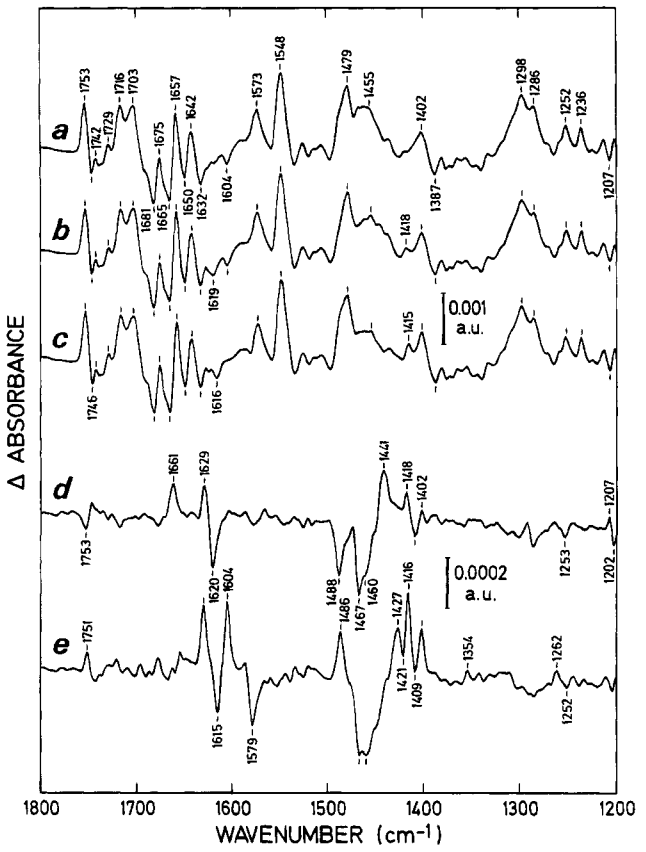

FIGURE 3: Light-induced $P^+Q_A^-/PQ_A$ FTIR difference spectra at 100 K of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with (a) unlabeled Q_3, (b) $^{13}C_1$-labeled Q_3, and (c) $^{13}C_4$-labeled Q_3 and double-difference spectra (^{13}C-labeled minus unlabeled) obtained for $^{13}C_1$-labeled Q_3 (d) and $^{13}C_4$-labeled Q_3 (e); average of 2 different pairs of samples.

vibration is unaffected by labeling at the 1-position. The positive bands at 1660 and 1628 cm^{-1} give rise to a single asymmetrical negative band of large amplitude at 1617 cm^{-1}. In the semiquinone absorption region of the double-difference spectra, the band at ~1484 cm^{-1} exhibits a negative sign upon $^{13}C_1$ labeling and a positive sign upon $^{13}C_4$ labeling. The main negative band at 1466 cm^{-1} appears to shift to 1440 or 1429 cm^{-1} upon $^{13}C_1$ or $^{13}C_4$ labeling, respectively, while a positive band at ~1418 cm^{-1} is seen for both labeling positions. Upon prolonged incubation of the quinone-reconstituted RCs into $H_2{}^{18}O$, the two quinone carbonyls are the only groups involved in the Q_A^-/Q_A spectra to become ^{18}O-labeled (Breton et al., 1994a). The double-difference spectra corresponding to the Q_A^-/Q_A spectra obtained for the $^{13}C_1$,^{18}O- and $^{13}C_4$,^{18}O-labeled Q_3 exhibit the three positive bands of the unlabeled quinone (Figure 2d′,e′). This is to be expected, as in this case each carbonyl bears at least one label. Double-difference spectra (Figure 3d,e) calculated for the $P^+Q_A^-/PQ_A$ spectra at 100 K of RCs reconstituted with either $^{13}C_1$- or $^{13}C_4$-labeled Q_3 and with unlabeled Q_3 show the same band pattern of the neutral and semiquinone forms as observed in the Q_A^-/Q_A double-difference spectra, with only minor upshifts (1−3 cm^{-1}) of the frequencies of the quinone bands.[2]

[2] The small signals in the 1740−1760-cm^{-1} frequency range (Figure 3d,e) are not considered as quinone contributions. They correspond to the absorption of the 10a-ester C=O of P and P^+, which varies slightly in $P^+Q_A^-/PQ_A$ spectra recorded on a given sample.

DISCUSSION

Isotope Effects on the Quinone Vibrations in Vitro. As previously discussed (Breton et al., 1994a,b), the splitting of the C=O bands at 1663 and 1650 cm^{-1} in the absorption spectrum of the isolated unlabeled Q_3 (Figure 1a) could have several origins. It is usually attributed either to Fermi resonance or to molecular asymmetry due to an inequivalence of the nature of the substituents. In the latter case, the two C=O modes could be split by inductive or resonance effects or simply because the mass of the substituents influences the vibrational coupling of the carbonyls to the quinone ring (Meyerson, 1985). The observation of a comparable splitting in the IR absorption spectrum of the symmetrical 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone (Breton et al., 1994b) rules out this possibility, while the presence of two C=O bands in the spectrum of fully ^{13}C-labeled Q_8 (Breton et al., 1994a) indicates that Fermi resonance is not likely to be responsible for the splitting. A third possibility would be that of a difference in the conformation of the two methoxy groups. Indeed, crystal structure studies (Silverman et al., 1971; Schmalle et al., 1984), electrochemical investigations (Prince et al., 1983), and quantum chemical calculations (Prince et al., 1988; Robinson & Khan, 1990; J.-R. Burie, unpublished results) have shown that adjacent methoxy groups in quinones adopt different geometries. The energy minimum for an unhindered methoxy group attached to a 1,4-benzoquinone ring corresponds to an orientation of the $O-CH_3$ bond away from the proximal carbonyl and coplanar with the quinone ring. When the quinone carries two adjacent methoxy groups, one $O-CH_3$ bond will adopt this geometry while the other will be out of the quinone plane with the $O-CH_3$ bond rotated by 120° (Silverman et al., 1971; Prince et al., 1988; Robinson & Khan, 1990; J.-R. Burie, unpublished results). By altering the delocalization of the p-electrons of the methoxy oxygen into the quinone ring, these geometrical differences modify the frequency of the carbonyl vibrations and provide a rationale for the 50% intensity decrease of both the 1663- and the 1650-cm^{-1} band of Q_3 *in vitro* upon labeling at either C_1 or C_4 (Figure 1a−c). For a given carbonyl, e.g., C_1, two IR bands corresponding to the in-plane and out-of-plane orientations of the proximal methoxy group are observed at 1663 and ~1650 cm^{-1}, and both bands downshift upon selective ^{13}C labeling. The same property also applies to the C_4 carbonyl.

The question then remains of the degree to which the 1663- and ~1650-cm^{-1} vibrations are coupled. Selective labeling of each carbonyl also provides information on this issue. If these were coupled C=O modes, one would expect the labeling of only one of the carbonyls to strongly influence the frequency of both modes. This is clearly not the case, as only minor frequency shifts are observed on the remaining bands at 1663 and ~1650 cm^{-1} after selective labeling of either carbonyl (Figure 1a−c). The conclusion that the vibration frequency of an unlabeled quinone carbonyl is only very slightly affected upon labeling of the opposite C=O group has also been reached in a recent study of partially ^{18}O-labeled quinones (Breton et al., 1994b).

Vibrations of Neutral Q_A in Rb. sphaeroides. In a previous FTIR study of the Q_A^-/Q_A spectra of *Rb. sphaeroides* RCs reconstituted with Q_6 labeled with ^{18}O on both carbonyls or with fully ^{13}C-labeled Q_8, three bands at 1660, 1628, and 1601 cm^{-1} were recognized as the C=O and C=C modes

Table 1: Frequency (cm^{-1}) and Assignment of Ubiquinone IR Bands in the Q_A Site of *Rb. sphaeroides* RCs[a]

	C_1=O			C=C			C_4=O		
		δ			δ			δ	
	freq	obsd	calcd	freq	obsd	calcd	freq	obsd	calcd
^{12}C,^{16}O-Q_3, -Q_6, -Q_8	1660			1628			1601		
^{12}C,^{18}O-Q_6	1625 ± 5	35 ± 5	40	1613	15	0	1586	15	39
^{13}C,^{16}O-Q_8	1628	42	37	1584	44	64	1545	56	36
^{13}C,^{18}O-Q_8	1583	77	78	1562	66	64	1540	61	75
$^{13}C_1$,$^{12}C_4$,^{16}O-Q_3	1623 ± 5	37 ± 5	37	1617	11	0	1601	0	0
$^{13}C_1$,$^{12}C_4$,^{18}O-Q_3	1585	75 [38]	78	1612	16 [5]	0	1585	16 [16]	39
$^{12}C_1$,$^{13}C_4$,^{16}O-Q_3	1660	0	0	1612	16	0	1578	23	36
$^{12}C_1$,$^{13}C_4$,^{18}O-Q_3	1626	36 [36]	40	1609	19 [3]	0	1548	53 [30]	75

[a] δ, isotopic shift; obsd = observed; calcd = calculated (using the harmonic oscillator approximation). Values in brackets correspond to the additional shift observed upon ^{18}O labeling of the selectively labeled Q_3.

of the neutral unlabeled Q_A (Breton et al., 1994a). The present experiments with Q_3 selectively labeled with ^{13}C at the 1- or the 4-position confirm the Q_A origin of these bands. This is further supported by the effect of ^{18}O labeling of selectively ^{13}C-labeled Q_3, which demonstrates the presence of the same three positive bands in the double-difference spectra for the ^{13}C,^{18}O-labeled minus unlabeled Q_A^-/Q_A spectra (Figure 2d′,e′). The 1660-cm^{-1} band has been assigned previously to one of the C=O modes of Q_A (Breton et al., 1994a). The present results show that the 1660-cm^{-1} band is unaffected by ^{13}C labeling at the 4-position and downshifts by 37 ± 5 cm^{-1} upon ^{13}C labeling at the 1-position, as expected for a pure C=O mode (Table 1). These observations provide compelling evidence for an assignment of the 1660-cm^{-1} band to the C_1 carbonyl of Q_A, i.e., to the carbonyl proximal to the isoprenoid chain.

In the previous investigation using nonspecifically labeled quinones, it was recognized that the two other Q_A bands at 1628 and 1601 cm^{-1} exhibit such a highly mixed C=O and C=C character that it was not possible to propose an assignment on the basis of the isotope effects alone. Nevertheless, taking into account the frequencies of the C=O modes (at 1663−1650 cm^{-1}) and the C=C mode (at 1611 cm^{-1}) of ubiquinone *in vitro*, the Q_A bands at 1628 and 1601 cm^{-1} were provisionally assigned to C=O and C=C modes, respectively (Breton et al., 1994a,b; see, however, the Note Added in Proof in the latter reference). The 1628-cm^{-1} band of Q_3 in the Q_A site of *Rb. sphaeroides* RCs shifts to 1617 cm^{-1} upon $^{13}C_1$ labeling and to 1612 cm^{-1} upon $^{13}C_4$ labeling (Table 1). In contrast, the 1601-cm^{-1} band is unaffected by the labeling of C_1 and is downshifted by 23 cm^{-1} upon labeling of C_4 (Table 1). This behavior of the 1601-cm^{-1} band indicates that the corresponding mode has predominantly C=O character. If the 1601-cm^{-1} band were a C=C mode coupled to the C=O modes, labeling should cause a downshift at both positions. Conversely, the shift of the 1628-cm^{-1} band by 11 and 16 cm^{-1} upon $^{13}C_1$ and $^{13}C_4$ labeling, respectively, is most compatible with its assignment to a C=C mode coupled to C=O modes. This assignment is further supported by the finding that ^{18}O labeling of selectively labeled Q_3 causes a larger additional frequency shift for the 1601- than for the 1628-cm^{-1} band (see Table 1 and Figure 2d′,e′).

The simplest interpretation of this new set of selective isotope labeling data is thus to reassign the 1628-cm^{-1} band to the C=C mode and the 1601-cm^{-1} band to the C_4=O mode of Q_A, keeping in mind that they both have a strongly mixed C=O and C=C character. This mixing makes the new assignment consistent with the isotopic shifts observed for the various labels which are in between those calculated for pure C=O or C=C stretching modes (Table 1). The assignment of the 1601-cm^{-1} band to the C_4=O mode implies that this mode is not coupled to the 1660-cm^{-1} C_1=O mode, as the labeling of one carbonyl has no effect on the frequency of the other. This uncoupled behavior of the C=O modes agrees with the observations on the ubiquinones *in vitro*.

The negative band at 1263 cm^{-1} in the Q_A^-/Q_A spectra (Figure 2a−c) has been assigned to C−O−C vibrations from the methoxy groups (Breton et al., 1994a,b). The absence of isotope effect on this band upon $^{13}C_1$ or $^{13}C_4$ labeling is consistent with this interpretation. The negative band at ~1373 cm^{-1} and the associated positive anion band at 1355 cm^{-1} also do not shift upon labeling of the two carbonyls (Figure 2a−c), which is consistent with their assignment to the δCH_3 vibration of the methyl group at the 5-position of the ring (Breton et al., 1994b).

Semiquinone Vibrations in Q_A^-. In the complex region of absorption of the C···O or C···C anion modes of Q_A^-, selective ^{13}C labeling of C_1 or C_4 leads to a considerable modification of all the bands in the Q_A^-/Q_A spectra (Figure 2a−c). This behavior of the anion modes is very different from that observed for the C=O and C=C modes of the neutral Q_A, for which the 1660- and 1601-cm^{-1} bands are selectively shifted only for one or the other of the two labeling positions. This indicates a definite coupling of the two C···O modes in Q_A^-. These modes are also coupled to the C···C modes, as previously inferred (i) from *ab initio* calculations (Chipman & Prebenda, 1986) and (ii) from the effect of ^{18}O labeling on the carbonyls of Q_6 and of full ^{13}C labeling of Q_8 (Breton et al., 1994a).

The anion band at 1484 cm^{-1} (Figure 2a) shifts to ~1420 cm^{-1} upon full ^{13}C labeling (Breton et al., 1994a) and thus cannot be due to a C···O mode, which should shift by at most 33 cm^{-1}. This band, which loses intensity upon ^{18}O labeling of both carbonyls and is sensitive to the nature of the side chain at the 6-position, has been proposed to contain predominantly C···C modes involving both the quinone ring and the C_6-C_{chain} mode (Breton et al., 1994a,b). This band essentially disappears upon $^{13}C_1$ labeling[3] (Figure 2b), while it is strongly enhanced upon $^{13}C_4$ labeling (Figure 2c). In addition, about two-thirds of this band shifts to 1466 cm^{-1} upon ^{18}O labeling of the $^{13}C_4$-labeled Q_3 (not shown). The complex behavior of this band illustrates the large coupling

of the motion of many atoms in Q_A^-. The Q_A^- mode at 1421 cm^{-1}, which increases in amplitude upon ^{18}O labeling of both carbonyls, has been tentatively assigned to a mode with predominant C···C character (Breton et al., 1994a). Although amplitude changes are observed around 1418 cm^{-1} for the Q_A^-/Q_A spectra obtained with $^{13}C_1$- or $^{13}C_4$-labeled Q_3, the overlap with the downshifted C···O modes for these labels prevents further insight into the precise nature of this mode.

The main anion band at 1466 cm^{-1} (Figure 2a) has been tentatively assigned to a mode with predominant C···O character (Breton et al., 1994a,b). Upon $^{13}C_1$ or $^{13}C_4$ labeling, this mode appears to shift to 1442 or 1430 cm^{-1}, respectively (Figure 2b,c). The 36-cm^{-1} shift observed upon $^{13}C_4$ labeling is too large for a pure C···O stretching mode. This suggests that the 1466-cm^{-1} band is not homogeneous and that the $^{12}C_4$···O mode might be slightly downshifted compared to the $^{12}C_1$···O mode. In this tentative assignment scheme, the two C···O modes of Q_A^- are both downshifted by at least 20 cm^{-1} compared to their frequency in tetrahydrofuran (Bauscher et al., 1990; Bauscher & Mäntele, 1992), in agreement with ENDOR data (Feher et al., 1985). In the absence of data on ubiquinone selectively labeled on the other carbon atoms of the quinone ring, these assignments for Q_A^- should only be considered as provisional.

Interaction of Q_A with the Protein. The clearest result emerging from the present study is obtained for the C_1=O vibration. This mode is responsible for the whole 1660-cm^{-1} band and is not coupled to the C_4=O mode. It is close in frequency to the carbonyls of Q_3 *in vitro* and is thus assigned to an essentially free carbonyl group. According to the X-ray structures of *Rb. sphaeroides* (Allen et al., 1988; El-Kabbani et al., 1991; Ermler et al., 1992), the C_1=O group, which is proximal to the isoprenoid chain, forms a hydrogen bond with the peptide NH of Ala M260 (*Rb. sphaeroides*). In the 2.8 Å resolution X-ray structure[4] of *Rb. sphaeroides* R-26 RC, the distance between the C_1 carbonyl oxygen and the peptide nitrogen is 2.83 Å, while the distance between the C_4 carbonyl oxygen and the other potential hydrogen bond donor (Thr M222) is 2.90 Å. The corresponding distances for the menaquinone-9 in *Rp. viridis* are 3.05 (with Ala M258) and 3.13 Å (with His M217), respectively (Michel et al., 1986; Deisenhofer & Michel, 1989). Thus, in both structures the stronger hydrogen bond appears to involve the interaction of the carbonyl proximal to the isoprenoid chain with the peptide NH of the Ala residue. The small differences in hydrogen bond distances are, however, below the precision of the X-ray data. In contrast to the conclusion derived from the X-ray studies, the present FTIR results appear to exclude a strong hydrogen bond to the C_1 carbonyl of ubiquinone in the Q_A site of *Rb. sphaeroides*.

While the C_1 carbonyl of Q_A appears little affected by the protein, a very different situation is encountered for the C_4 carbonyl. The Q_A^-/Q_A FTIR difference spectrum of selectively $^{13}C_4$-labeled Q_3 shows a very large perturbation of the C=O and C=C modes associated with this position. In our present interpretation of the FTIR data, the C_4=O vibration is well localized at 1601 cm^{-1} with a strong admixture of C=C character, and the C=C mode is found at 1628 cm^{-1} with a strong contribution from C=O character. These observations point to an unusual environment of the C_4 carbonyl *in vivo*. Although quinones *in vitro* are rather insensitive to the hydrogen-bonding properties of the solvent, large downshifts of their carbonyl frequencies have been reported under special conditions leading to the formation of strong hydrogen bonds, such as complexation of quinone with dihydroquinone (thus forming the quinhydrone), for which shifts of 20−30 cm^{-1} have been observed (Slifkin & Walmsley, 1970; Kruk et al., 1993). Downshifts of 30−80 cm^{-1} have been reported upon intramolecular hydrogen bonding interactions in β-hydroxylated naphthoquinones and anthraquinones (Hadži & Sheppard, 1954; Bloom et al., 1959). In line with the 70−180-cm^{-1} downshifts documented for Lewis acids interacting with carbonyl groups, notably with phenyl ketones (Bellamy, 1980), it has been found that complexation of quinones with trichloroacetic acid or with $AlCl_3$ (in a 1:1 ratio) can cause downshifts by 30−70 cm^{-1} of the frequency of only one of the carbonyl groups (J.-R. Burie, unpublished results). Compared to the frequency found *in vitro*, the ~50−60-cm^{-1} downshift of the C_4=O mode of ubiquinone observed upon binding to the protein is thus not unprecedented and would correspond to a very strong hydrogen bond, of the order of −6 to −8 kcal mol^{-1} (Badger & Bauer, 1937; Breton et al., 1994a). Such strong hydrogen bonding is often achieved in conjugated chelation systems when resonance involving delocalized charges comes into play (Bloom et al., 1959). In this respect it is worth noting that, in the highest resolution X-ray structure available at present for *Rb. sphaeroides* (Ermler et al., 1992), the C_4 carbonyl is positioned in hydrogen-bonding interaction with the proton (at Nδ1) of the His M219 residue (Figure 4), which is also a ligand (at Nϵ2) of the non-heme Fe^{2+} atom. This positioning could favor electronic resonance within the conjugated imidazole ring. The assignment of the 1601-cm^{-1} band to a strongly hydrogen bonded carbonyl of Q_A agrees with binding affinity studies, which indicate that quinones in the Q_A site form only one strong hydrogen bond with a binding free energy of −3 to −7 kcal mol^{-1} (Gunner et al., 1985; Warnke & Dutton, 1993; Warnke et al., 1994). However, in view of the unusual response of the quinones to hydrogen bond donors in solution, it is possible that the distances between the C_1=O and C_4=O oxygen atoms and their respective donors proposed in the X-ray structures are indeed close to their actual values but that effects other than the mere distance determine the frequency of the carbonyl vibrations.

Comparison with ^{13}C NMR Results. Our assignment scheme contrasts with the conclusions from NMR studies indicating that the C_4 atom is subject to considerable heterogeneity at temperatures above 230 K (van Liemt, 1994). This observation was interpreted as suggesting the mobility of a nearby residue and was thus considered not to support the notion of a strong hydrogen bond to the C_4 carbonyl. In this respect, it is significant that the 1601-cm^{-1} mode is almost unaffected by temperature, as it appears at 1604 cm^{-1} in $P^+Q_A^-/PQ_A$ FTIR difference spectra recorded either at 100 K (Figure 3; Bagley et al., 1990; Nabedryk et

[3] With the possible exception of a very small signal at ~1470 cm^{-1}, the residual absorption in the 1480−1430-cm^{-1} frequency range (Figure 2b) cannot originate from the downshifted 1484-cm^{-1} band. This background absorption is observed in the Q_A^-/Q_A spectra of fully ^{13}C,^{16}O- and ^{13}C,^{18}O-labeled Q_8 (Breton et al., 1994a, and data not shown) and thus most probably originates from the protein.

[4] Entry 4RCR in the Brookhaven Protein Data Bank.

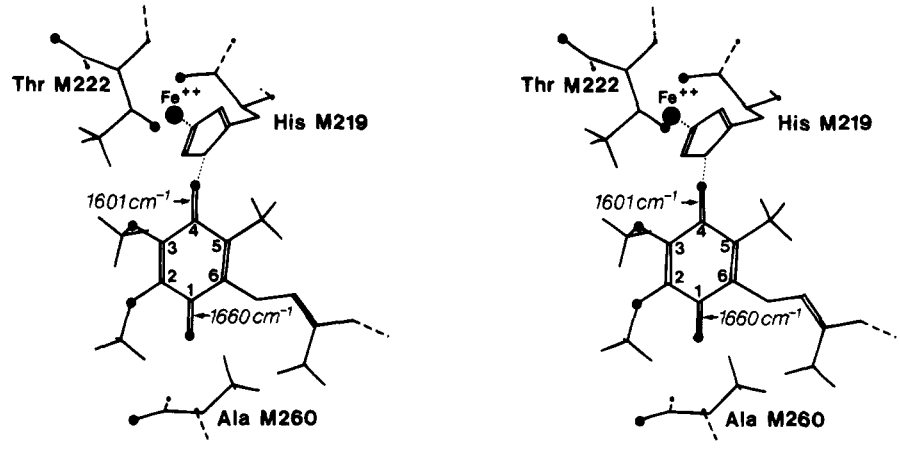

FIGURE 4: Schematic stereoview of the interaction of Q_A with the protein binding site of *Rb. sphaeroides* RC as derived from X-ray crystallography [Allen et al., 1988 (entry 4RCR in the Brookhaven Protein Data Bank); Ermler et al., 1992] and from the present FTIR study. The C−H bonds are depicted only for the methyl groups. Filled circles: small for nitrogen, medium for oxygen, and large for iron. Broken lines indicate the truncation of the polypeptide or isoprenyl chains.

al., 1990) or at 10 K (J. Breton, unpublished results). The origin of the discrepancy between the FTIR and NMR results cannot be due to an effect of the difference in the length of the side chains of the ubiquinones used in the two studies, as the Q_A^-/Q_A FTIR difference spectrum recorded with RCs reconstituted with unlabeled Q_3 is indistinguishable from that of untreated RCs containing the native Q_{10}. Thus, this discrepancy probably reflects some physical property that influences differently the measurements by these two techniques. It might be traceable to an electrostatic effect from the protein binding site, such as the atomic charges of Fe^{+2} or the histidine residue that probably is the hydrogen bond donor to the C_4 carbonyl. The crystal structure of *Rb. sphaeroides* RCs[4] shows that the Fe^{2+} is 9.2 Å from C_4 compared to 10.9 Å for C_1. The effect on the NMR signal of the coupling of the electronic spin of the paramagnetic Fe^{2+} with the nuclear spin of the ^{13}C atom scales with r^{-6}, and is thus ~3 times larger for C_4 than for C_1. This would be consistent with a broadening of the $^{13}C_4$ NMR signal, which would make it unresolvable from the rather high background noise (van Liemt, 1994). As the $^{13}C_4$ NMR signal appears upon decreasing the temperature, this suggests a dynamic rather than a static dipolar interaction effect. It may thus be relevant that solid-state NMR probes the Q_A environment on the millisecond time scale, while IR spectroscopy samples the RC population on a subpicosecond time scale. Dynamic processes occurring in the time window between these two domains will affect the two types of measurements differently. While the authors of the NMR work favor motion of a residue close to the C_4 carbonyl (van Liemt et al., 1993; van Liemt, 1994), other dynamic effects should also be considered. For example, a small fluctuation of the distance between the neutral quinone and Fe^{2+} would broaden the NMR signal from the C_4 atom more than that from C_1. The presence of a bifurcated hydrogen bond between the C_4 carbonyl and the protein (e.g., with His M219 *and* Thr M222), which is excluded neither by the X-ray data nor by the present FTIR results, could lead to a shifting electron density between the two hydrogen bonds that would make the $^{13}C_4$ atom NMR-silent. Alternatively, rocking of the methoxy groups between two conformations could have more influence on the solid-state NMR signal than on the FTIR spectrum. These motions would freeze with the solvent, thus allowing the NMR signal from the $^{13}C_4$ atom to be detected below 250 K.

Absence of Displacement of Q_A upon Reduction. The striking similarity of the double-difference spectra obtained at 278 K (Figure 2d,e) and at 100 K (Figure 3d,e) provides information on the possibility that Q_A moves upon photoreduction. Such a movement has been proposed on the basis of X-ray data (Allen et al., 1988), EPR experiments (Calvo et al., 1990), molecular dynamics simulations (Nonella & Schulten, 1991), and Q_A^-/Q_A FTIR spectra (Bauscher et al., 1993). The distance between Q_A and Fe^{2+} would decrease upon photoreduction by 0.4 Å in *Rp. viridis* (Nonella & Schulten, 1991) and by 1.6 Å in *Rb. sphaeroides* RCs (Calvo et al., 1990). The double-difference spectra (Figures 2d,e and 3d,e) represent a very sensitive molecular fingerprint of the interactions of Q_A with the protein in both the neutral and the semiquinone state. Thus, the close similarity of the double-difference spectra measured at 100 and 278 K demonstrates that any movement of the quinone relative to its protein binding site which could take place upon photoreduction is the same at both temperatures. Furthermore, the near identity of the $P^+Q_A^-/PQ_A$ spectra at 100 and 10 K in the region of absorption of the carbonyls of Q_A and Q_A^-, notably with the presence at 10 K of the 1604-cm^{-1} band diagnostic of the strong interaction of the C_4 carbonyl with the protein (J. Breton, unpublished results), shows this property of the Q_A−RC complex to be also valid at 10 K. Reduction of quinones *in vitro* is accompanied by bond length increase and decrease by ~0.05 Å of the C=O and C=C double bonds and of the C−C single bonds, respectively (Chipman & Prebenda, 1986; Robinson & Kahn, 1990). The closeness of the double-difference spectra at 278 and 100 K (Figures 2d,e and 3d,e) and of the $P^+Q_A^-/PQ_A$ spectra at 100 and 10 K shows that such changes are indeed occurring and that they are the same at all temperatures. On the other hand, it is most probable that the whole Q_A molecule cannot move significantly out of its binding site at the cryogenic temperatures. Thus, the FTIR results are taken to show that, if the distance between Fe^{2+} and Q_A does decrease upon reduction, this movement involves no significant displacement of the center of mass of the quinone with respect to its protein binding site.

In conclusion, the present results have allowed the identification of the quinone carbonyl proximal to the isoprenoid chain and close to the peptide NH of Ala M260 as a group essentially free from interaction with the protein binding site. For the carbonyl proximal to the methyl group, the FTIR results demonstrate that this C=O is highly perturbed, exhibiting an extensive frequency downshift and a drastic alteration of the coupling of the C=O and C=C modes. This perturbation, probably related to the combination of a strong hydrogen bond with His M219 and an effect of electronic coupling with the Fe^{2+} atom through the imidazole ring of the histidine residue, is currently under FTIR investigation of Q_A^-/Q_A spectra using Zn-substituted RCs, mutants at the Q_A site, and other isotopically labeled quinones.

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