Asymmetric binding of the 1- and 4-C=0 of QA in Rhodobacter sphaeroides R26 reaction centres monitored by Fourier transform infra-red spectroscopy using site-specific isotopically laballed ubiquinone-10

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Asymmetric binding of the 1- and 4-C=O groups of Q_A in *Rhodobacter sphaeroides* R26 reaction centres monitored by Fourier transform infra-red spectroscopy using site-specific isotopically labelled ubiquinone-10

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Using 1-, 2-, 3- and 4-^{13}C site-specifically labelled ubiquinone-10, reconstituted at the Q_A site of *Rhodobacter sphaeroides* R26 reaction centres, the infra-red bands dominated by the 1- and 4-C=O vibration of Q_A are assigned in the $Q_A^- - Q_A$ difference spectra. The mode dominated by the 4-C=O vibration is drastically downshifted in the reaction centres as compared with its absorption frequency in free ubiquinone-10. In contrast, the mode dominated by the 1-C=O vibration absorbs at similar frequencies in the free and the bound forms. The frequency shift of the 4-C=O vibration is due to a large decrease in bond order and indicates a strong interaction with the protein microenvironment in the ground state. In the charge-separated state the mode dominated by the semiquinone 4-C⋯O vibration is characteristic of strong hydrogen bonding to the microenvironment, whereas the mode dominated by the 1-C⋯O vibration indicates a weaker interaction. The asymmetric binding of the 1- and 4-C=O groups to the protein might contribute to the factors governing different redox reactions of ubiquinone-10 at the Q_A site as compared with its reactions at the Q_B site.
Key words: bacterial reaction centres/electron transfer/ Fourier transform infra-red/isotopic labelling/photosynthesis/ubiquinone

Introduction

Understanding photosynthesis at the atomic level is a challenge in structural biology today. The primary event of photosynthesis is a light-induced transmembrane charge separation, occurring in a pigment protein complex termed the reaction centre (RC). The ground state structures of the RCs of two purple bacteria, *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides*, have been resolved by X-ray crystallography with nearly atomic resolution (Deisenhofer and Michel, 1989; Feher *et al.*, 1989; Chang *et al.*, 1991). Even though this represents a fundamental step in the understanding of the function of RCs, to achieve a complete picture their dynamic behaviour during charge separation has to be determined at the atomic level by time-resolved methods. Redox reactions of the prosthetic groups, monitored by absorption changes in the visible spectral range, have been determined with a time resolution down to fs (Kirmaier and Holten, 1987; Boxer, 1990). These results provide a detailed description of the electron transfer kinetics, originating at the primary electron donor P (bacteriochlorophyll dimer), proceeding via the intermediary electron acceptor I (bacteriopheophytin) and the primary electron acceptor quinone Q_A, and terminating at the secondary electron acceptor quinone Q_B. The accessory bacteriochlorophyll between P and I is reported to be a real electron carrier too (Arlt *et al.*, 1993).

Fourier transform infra-red (FTIR) spectroscopy has been applied to monitor not only the chromophore but also the protein backbone and side-chain reactions at an atomic level (Gerwert, 1993). High quality infra-red difference spectra of individual charge-separated states have been obtained for *Rps.viridis* and *Rb.sphaeroides* (Mäntele, 1993). At first, the $P^+Q_A^- - PQ_A$ and $P^+Q_B^- - PQ_B$ difference spectra have been recorded (Mäntele *et al.*, 1985; Gerwert *et al.*, 1988; Nabedryk *et al.*, 1990). The observed band pattern is typical for oxidation of bacteriochlorophyll in a hydrophobic environment (Gerwert *et al.*, 1988). Large-scale structural changes during charge separation can be excluded, indicating that the protein matrix surrounding the chromophores remains rigid throughout electron transfer and that the ground state structure also seems a reasonable structural model for the charge-separated states (Mäntele *et al.*, 1985; Gerwert *et al.*, 1988). The $P^+Q^- - PQ$ difference spectra are dominated by the P^+/P vibrations that tend to mask the quinone absorption changes (Mäntele *et al.*, 1985; Bagley *et al.*, 1990; Leonhard and Mäntele, 1993). Therefore, in another approach by adding reductants which rapidly re-reduce the primary donor, $I^- - I$ and $I^-Q_A^- - IQ_A$ difference spectra have been obtained allowing the identification of semiquinone vibrations (Buchanan *et al.*, 1990, 1992). The tentatively assigned semiquinone absorption bands indicate hydrogen bonding of semiquinone (Buchanan *et al.*, 1992). Tentative assignments based on the frequencies of the absorption bands to quinone, protein side group and backbone vibrations have been reported (Thibodeau *et al.*, 1990; Breton *et al.*, 1991a,b, 1992; Hienerwadel *et al.*, 1992; Thibodeau *et al.*, 1992; Bauscher *et al.*, 1993). But bands of these groups absorb in overlapping spectral regions; in addition, they may have unusual frequency shifts in the protein environment as compared with spectra of model compounds. Therefore these assignments are ambiguous.
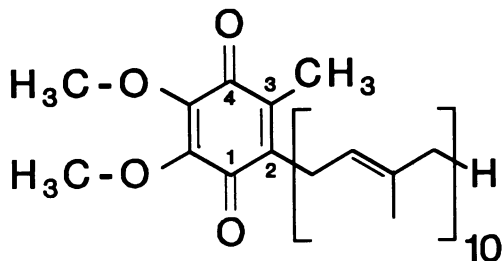
Fig. 1. Ubiquinone-10.

To draw quantitative conclusions from the spectra, clear-cut assignments based on site-specific isotopic labelling or site-specific mutation have to be performed as a prerequisite. Here we present for the first time $Q_A^- - Q_A$ difference spectra of *Rb.sphaeroides* reconstituted at the Q_A site with ubiquinone-10 (UQ_{10}) specifically ^{13}C-labelled at positions 1–4, respectively (Figure 1). This now allows clear-cut assignments of the 1- and 4-C=O and 2- and 3-C=C vibrations of Q_A in the neutral and the charge-separated states. The implications of these results for the mechanism of binding of UQ_{10} at the Q_A site to the protein environment, and for the fast-forward electron transfer rates as compared with the recombination rates, will be discussed.

Results

Vibrations of UQ_{10}

Figure 2a shows the infra-red absorption spectrum of UQ_{10} between 1700 and 1550 cm^{-1} in the region of the C=O and C=C stretching vibrations. The spectrum agrees nicely with the UQ_{10} spectrum in CCl_4 (Breton *et al.*, 1992). In Figure 2b and c the spectra of 1- and 4-^{13}C-labelled UQ_{10} are shown. The good agreement of Figure 2b and c is remarkable, although UQ_{10} is labelled at different positions. Only small intensity variations are seen at 1620 and 1600 cm^{-1} comparing Figure 2b and c. In the carbonyl region in both spectra the bands at 1666 and 1650 cm^{-1} decrease in intensity and a new band appears at 1620 cm^{-1}. Furthermore, the band at 1611 cm^{-1} loses intensity and is partially shifted to 1600 cm^{-1}. The general similarity of 1- and 4-^{13}C-labelled UQ_{10} spectra indicates the equivalence of positions 1 and 4 in the free UQ_{10}.

In Figure 2d and e the spectra of 2- and 3-^{13}C-labelled UQ_{10} are shown; they also agree. The bands at 1666 and 1650 cm^{-1} are slightly less intense as compared with the unlabelled compound. A new band is observed at 1638 cm^{-1}. This band is less shifted as compared with the bandshift to 1620 cm^{-1} due to 1 and 4 labelling. The band at 1611 cm^{-1} is completely shifted to 1596 cm^{-1}. As for positions 1 and 4, the great similarity of the 2- and 3-^{13}C-UQ_{10} spectra indicates the equivalence of positions 2 and 3 in free UQ_{10}.

Vibrations of UQ_{10} bound at the Q_A site of *Rb.sphaeroides* RCs

To assign the carbonyl vibrations of UQ_{10} bound in the *Rb.sphaeroides* RC, $Q_A^- - Q_A$ difference spectra have been recorded. The difference between the ground state and the charge-separated state allows the selection of
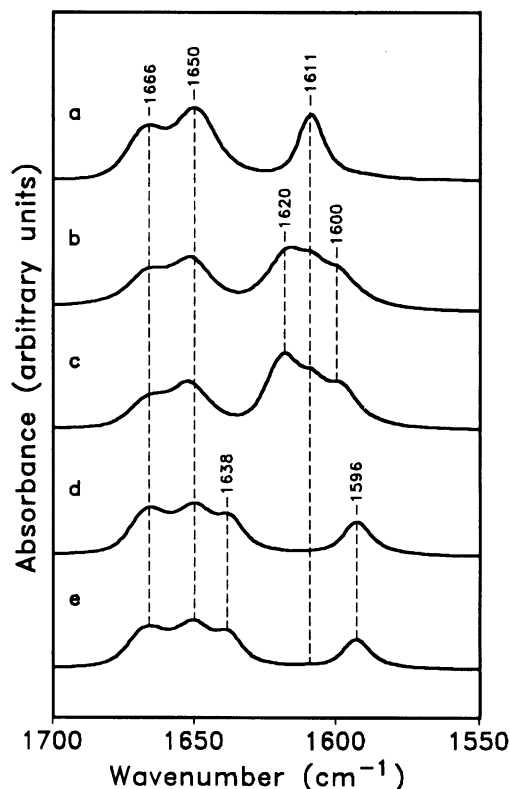

Fig. 2. Infra-red spectra of free ubiquinone-10 in the region of the C=O and C=C stretching vibrations. (a) Unlabelled; (b) 1-^{13}C-labelled; (c) 4-^{13}C-labelled; (d) 2-^{13}C-labelled; (e) 3-^{13}C-labelled.

absorption bands of those groups undergoing reactions out of the quiescent background absorption of the whole protein. Negative bands in the difference spectra belong to the ground state and positive bands to the charge-separated state absorption. By comparing $Q_A^- - Q_A$ difference spectra of unlabelled with site-specific ^{13}C-labelled UQ_{10}, quinone absorption bands are assigned. Isotopic labelling shifts the labelled group frequency to lower wave numbers.

In Figure 3a the $Q_A^- - Q_A$ difference spectrum of *Rb.sphaeroides* RCs reconstituted with unlabelled UQ_{10} is shown. The same reconstitution procedure was applied to the unlabelled reference samples to avoid systematic errors introduced by the reconstitution method. The $Q_A^- - Q_A$ difference spectrum agrees remarkably well with the one presented by Breton *et al.* (1991a) with the following exception: the band pattern between 1650 and 1630 cm^{-1} has less intensity in Breton *et al.* (1991a), and an additional broad negative band seems to decrease the intensity of the band at 1486 cm^{-1} in the difference spectrum presented here. Deviations of intensities in the water OH bending vibration region at 1640 cm^{-1} might be caused by different water content of the samples. Due to extensive experience with time-resolved measurements on bacteriorhodopsin (Heßling *et al.*, 1993), great care was taken to hydrate the sample fully. As a control of sufficient water content, the rate constants of $P^+Q_A^-$ decay of the wet infra-red sample have to match the rate constants of the sample in suspension. Insufficient water content slows down the rates in the infra-red sample. The variation at 1486 cm^{-1} as compared with Breton *et al.* (1991a) could be caused
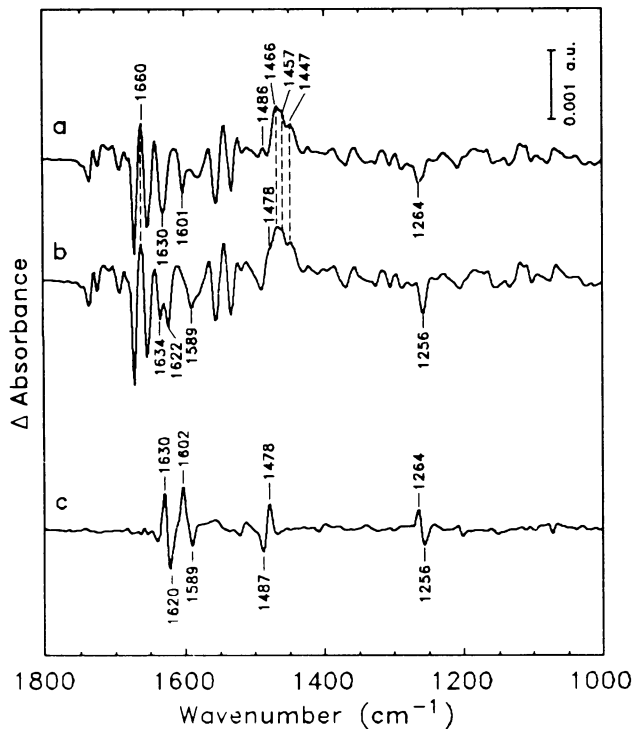
Fig. 3. Q$_A^-$−Q$_A$ difference spectra of *Rb.sphaeroides* RCs reconstituted with (**a**) unlabelled and (**b**) 3-^{13}C-labelled UQ$_{10}$ at the Q$_A$ site. (**c**) Double difference spectrum (b–a).

Fig. 4. Q$_A^-$−Q$_A$ difference spectra of *Rb.sphaeroides* RCs reconstituted with (**a**) unlabelled and (**b**) 2-^{13}C-labelled UQ$_{10}$ at the Q$_A$ site. (**c**) Double difference spectrum (b–a).

by the different sample treatment. Bauscher *et al.* (1993) also presented Q$_A^-$−Q$_A$ difference spectra achieved by three different methods. They deviate when compared with each other. Compared with the difference spectra presented here and in Bauscher *et al.* (1993), the electrochemically generated one shows general similarity except in the region between 1670 and 1650 cm^{-1}.

In Figure 3b the Q$_A^-$−Q$_A$ difference spectrum of RCs reconstituted with 3-^{13}C-labelled UQ$_{10}$ is shown. In the difference spectrum the negative band at 1630 (Figure 3a) is partially shifted to 1622 cm^{-1} (Figure 3b). To quantify the deviations between both difference spectra, double difference spectra, as described in Materials and methods, were performed (Figure 3c). The shift of a negative band from 1630 to 1622 cm^{-1} is indicated in the double difference spectrum by a positive band at 1630 and a negative band at 1620 cm^{-1}. Furthermore, the band at 1601 (Figure 3a) is shifted to 1589 cm^{-1} (Figure 3b). This is mirrored in the double difference spectrum in Figure 3c by a positive band at 1602 and a negative band at 1589 cm^{-1}. The intensity of the shifted band at 1589 cm^{-1} should be the same as that for the band at 1602 cm^{-1}. The reason for the missing intensity is not yet clear. In the semiquinone region a positive band is shifted from 1486 (Figure 3a) to 1478 cm^{-1} (Figure 3b). This is visualized in the double difference spectrum by a negative band at 1487 and a positive band at 1478 cm^{-1}. The real size of the band at 1486 cm^{-1} is masked by an underlying negative band. The underlying negative band at 1486 cm^{-1} is clearly seen in Figure 3b. The band at 1264 is shifted to 1256 cm^{-1}, as seen in the double difference spectrum by a positive band at 1264 and a negative band at 1256 cm^{-1}.

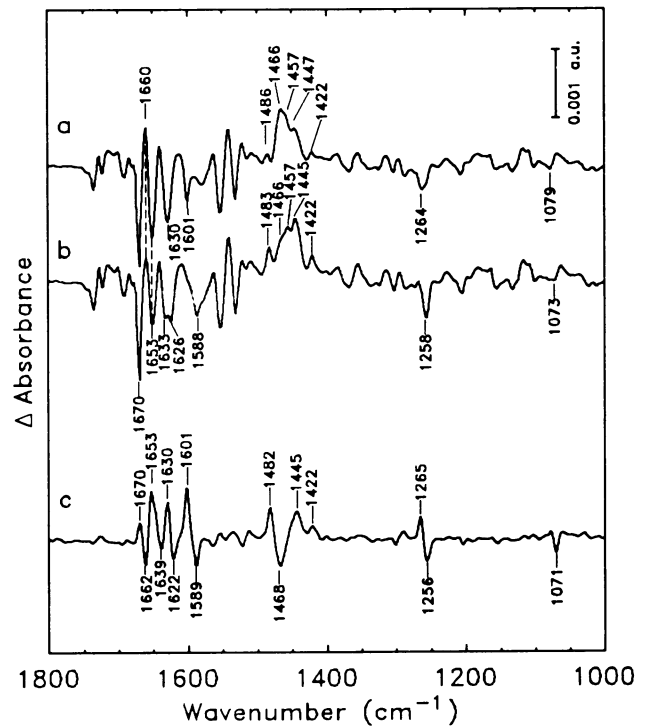In Figure 4 the Q$_A^-$−Q$_A$ difference spectra of unlabelled

(Figure 4a) and 2-^{13}C-labelled UQ$_{10}$ (Figure 4b) and the corresponding double difference spectrum (Figure 4c) are shown. It is obvious that the difference spectra of 2- and 3-^{13}C-labelled quinones are no longer similar as in the free compound (compare Figures 3b and 4b with Figure 2d and e). Therefore, positions 2 and 3 of UQ$_{10}$ embedded in the protein matrix are, in contrast to the free compound, no longer equivalent. The 2-^{13}C labelling induces several bandshifts. The intensity variations at ~1670/1660 cm^{-1} in Figure 4b as compared with Figure 4a are reflected by the 1670/1662 cm^{-1} difference band in the double difference spectrum in Figure 4c. The intensity variation at ~1670 cm^{-1} is seen more or less in all difference spectra, but is not specific to one label. This may indicate a global label effect. A broad negative band at ~1653 cm^{-1} is shifted to 1639 cm^{-1}, leading to a decreased intensity at 1653 cm^{-1} in Figure 4b as compared with Figure 4a. This is seen as a difference band at 1653/1639 cm^{-1} in the double difference spectrum. Part of the negative band at 1630 (Figure 4a) is shifted to 1626 cm^{-1} (Figure 4b) in the double difference spectrum indicated by the 1630/1622 cm^{-1} difference band. The positive band at 1630 cm^{-1} in the double difference spectrum partially cancels the intensity of the negative band at 1639 cm^{-1}. The band at 1601 in Figure 4a is shifted to 1589 cm^{-1} in Figure 4b, visualized by the 1601/1589 cm^{-1} difference band in Figure 4c. The band at 1486 cm^{-1} gains intensity in Figure 4b, indicating that a negative band is shifted. This is reflected by a positive band at 1482 cm^{-1} in Figure 4c. The band seems shifted to ~1468 cm^{-1}, indicated by a negative band at 1468 cm^{-1} in Figure 4c. The band at 1466 is shifted to 1445 cm^{-1} (compare Figure 4a and b), also seen as a difference band at 1468/1445 cm^{-1} in the double difference spectrum.

Fig. 5. $Q_A^- - Q_A$ difference spectra of *Rb.sphaeroides* RCs reconstituted with (**a**) unlabelled and (**b**) 4-^{13}C-labelled UQ_{10} at the Q_A site. (**c**) Double difference spectrum (b–a).

Fig. 6. $Q_A^- - Q_A$ difference spectra of *Rb.sphaeroides* RCs reconstituted with (**a**) unlabelled and (**b**) 1-^{13}C-labelled UQ_{10} at the Q_A site. (**c**) Double difference spectrum (b–a).

The integral intensity of the band at 1468 cm^{-1} in Figure 4c is larger than the integral intensity of the bands at 1482 and 1445 cm^{-1}, indicating that it represents a shifted negative band from 1486 cm^{-1} and a shift of a positive band from 1466 cm^{-1}. Another positive band in this spectral region (most probably the band at 1447 cm^{-1}) must be shifted because the band at 1422 cm^{-1} gains intensity. Additional shifts are seen at 1264–1258 and at 1079–1073 cm^{-1}, also reflected as difference bands in the double difference spectrum.

In Figure 5 the $Q_A^- - Q_A$ difference spectra of unlabelled (Figure 5a) and 4-^{13}C-labelled UQ_{10} (Figure 5b) and the corresponding double difference spectrum (Figure 5c) are shown. Again, small intensity changes are seen at 1670/1660 cm^{-1} (compare Figure 5a and b). A part of the band at 1630 (Figure 5a) is shifted to 1612 cm^{-1} (Figure 5b), indicated by the 1628/1613 cm^{-1} difference band in the double difference spectrum. The band at 1601 (Figure 5a) is shifted to 1579 cm^{-1} (Figure 5b), reflected by the 1601/1579 cm^{-1} difference band (Figure 5c). At 1486 cm^{-1} (Figure 5a) a negative band is shifted, as for 2-^{13}C labelling, to ~1466 cm^{-1}. The bands at 1466 and 1447 cm^{-1} (Figure 5a) are most probably shifted to 1430 and 1418 cm^{-1} and a positive band to 1401 cm^{-1} (Figure 5b). These bandshifts are reflected in the double difference spectrum (Figure 5c) by the broad negative band at 1466/1449 cm^{-1} and positive bands at 1430, 1418 and 1401 cm^{-1}.

In Figure 6 the $Q_A^- - Q_A$ difference spectra of unlabelled (Figure 6a) and 1-^{13}C-labelled UQ_{10} (Figure 6b) and the corresponding double difference spectrum (Figure 6c) are shown. A negative band at 1660 cm^{-1} is shifted to lower wave numbers, indicated by an intensity increase at 1660 cm^{-1} in Figure 6b as compared with Figure 6a. This is

reflected by a positive band in the double difference spectrum at 1660 cm^{-1}. The shifted, negative band is not seen in the double difference spectrum. Part of the negative band at 1630 (Figure 6a) is shifted to 1618 cm^{-1} (Figure 6b). In the double difference spectrum the integral intensity of the positive band at 1628 cm^{-1} is less compared with the intensity of the negative band at 1618 cm^{-1}. This can be explained by a shift of the band from 1660 cm^{-1} underneath the band at 1628 cm^{-1} in Figure 6c. The most likely explanation is a shift of the band at 1660 to ~1630 cm^{-1}. In the semiquinone vibration region the band at 1486 is shifted to 1470 cm^{-1} (compare Figure 6a and b), indicated by the 1486/1479 cm^{-1} difference band in Figure 6c. The bands at 1466 and 1447 cm^{-1} are shifted to 1443 and 1421 cm^{-1} (compare Figure 6a and b). This is reflected by the negative band at 1466 and positive bands at 1441 and 1420 cm^{-1} (Figure 6c). The positive band at 1470 cm^{-1} is partially masked by the overlapping negative band at 1466 cm^{-1} (Figure 6c). At 1447 cm^{-1} the negative band is masked by the shifted positive band from 1466 cm^{-1}, indicated by the larger integral intensity at 1466 as compared with 1441 cm^{-1}.

Discussion

The isotopic shifts observed on free UQ_{10} are summarized in Table I. For a complete quantitative assignment of the bands to internal vibrations, a normal mode analysis has to be performed. Nevertheless, the strong isotopic shifts observed allow the assignment of the dominant internal vibration to the normal mode representing the respective absorption band. Isotopic labelling at C=O positions 1 and 4 and C=C positions 2 and 3 induces frequency shifts of the bands at 1666, 1650 and 1611 cm^{-1}, indicating

Table I. Absorption frequencies (cm^{-1}) of unlabelled and 1-^{13}C-, 2-^{13}C-, 3-^{13}C- and 4-^{13}C-labelled UQ$_{10}$ between 1700 and 1550 cm^{-1}

UQ$_{10}$	1-^{13}C/4-^{13}C	2-^{13}C/3-^{13}C	Assignment of dominant vibration
1666/1650	partially to 1620 (−46/−30)	partially to 1638 (−28/−12)	1/4-C=O
1611	partially to 1600 (−11)	1596 (−15)	2/3-C=C

The frequency shifts caused by the labels are indicated in brackets.

Table II. Absorption frequencies (cm^{-1}) of unlabelled and 1-^{13}C-, 2-^{13}C-, 3-^{13}C- and 4-^{13}C-labelled UQ$_{10}$ between 1700 and 1550 cm^{-1} at the Q$_A$ site of *Rb.sphaeroides* RCs

Q$_A$	1-^{13}C	4-^{13}C	2-^{13}C	3-^{13}C	Assignment of dominant vibration
1660	~1630 (−30)	−	~1639 (−21)	−	1-C=O
1628	1618 (−10)	1613 (−15)	1622 (−6)	1620 (−8)	C=O/C=C
1601	−	1579 (−22)	1589 (−12)	1589 (−12)	4-C=O

The frequency shifts caused by the labels are indicated in brackets.

Table III. Absorption frequencies (cm^{-1}) of unlabelled and 1-^{13}C-, 2-^{13}C-, 3-^{13}C- and 4-^{13}C-labelled UQ$_{10}$ between 1500 and 1400 cm^{-1} at the Q$_A$ site of *Rb.sphaeroides* RCs in the semiquinone state

Q$_A{}^-$	1-^{13}C	4-^{13}C	2-^{13}C	3-^{13}C	Assignment of dominant vibration
1486	1470 (−16)	−	−	1478 (−8)	1-C⸚O
1466	1441 (−25)	1430 (−36)	1445 (−21)	−	4-C⸚O
1447	1420 (−27)	1418 (−29)	1422 (−25)	−	C⸚C

The frequency shifts caused by the labels are indicated in brackets.

strong coupling between the C=O and C=C ring stretching vibrations. The larger shifts of the bands at 1666 and 1650 cm^{-1} due to labelling at positions 1 and 4 compared with the shifts of these bands due to labelling at positions 2 and 3 show a dominant contribution from the 1- and 4-carbonyl stretching vibrations to the modes causing bands at 1666 and 1650 cm^{-1}. Nevertheless, the 1- and 4-carbonyl vibrations cannot be assigned individually to one of the bands at 1666 and 1650 cm^{-1} because both positions seem to be equivalent in the free UQ$_{10}$ and the vibrations mix with each other. On the other hand, the band at 1611 is completely shifted to 1596 cm^{-1} by labelling at positions 2 and 3, but is less affected by labelling at positions 1 and 4. This indicates a dominant contribution of the 2/3-C=C ring stretching vibrations to the band at 1611 cm^{-1}.

The isotopic shifts observed for UQ$_{10}$ bound at the Q$_A$ site of *Rb.sphaeroides* in the C=O and C=C stretching vibration regions are summarized in Table II. As in the free UQ$_{10}$, the C=C and C=O vibrations are strongly coupled, but dominant character can be attributed and the C=O vibrations are not mixed as in free UQ$_{10}$. The band at 1660 cm^{-1} is shifted only by 1- (Δv ≈ −30 cm^{-1}) and 2-^{13}C (Δv ≈ −21 cm^{-1}) labelling and is therefore dominated by the 1-C=O vibration without contribution of the 4-C=O vibration (Table II). On the other hand, the band at 1601 cm^{-1} is shifted by 4- (Δv = −22 cm^{-1}) and less by 2- and 3-^{13}C (Δv = −12 cm^{-1}) labelling (Table II). The mode is mainly composed of the 4-C=O vibration without contribution of the 1-C=O vibration, but is strongly coupled to the 2/3-C=C vibration. The band at 1628 cm^{-1} is shifted by all four labels (see Table II) and represents a mode with highly coupled 1-C=O, 4-C=O and 2/3-C=C vibrations. It is surprising to find the mode of the coupled C=O/C=C vibrations at a higher frequency than the mode that is dominated by the 4-C=

O vibration. In contrast to earlier tentative assignments (Bauscher *et al.*, 1993), attribution of the whole band at 1630 cm^{-1} to both C=O vibrations is excluded by the results presented here. Even though the band at 1630 cm^{-1} is partially due to the 1- and 4-C=O vibrations, the mode dominated by the 1-C=O group is identified to absorb at 1660 cm^{-1} and the mode dominated by the 4-C=O group is seen at 1601 cm^{-1}.

Semiquinone vibrations are expected between 1500 and 1400 cm^{-1} (Bauscher *et al.*, 1990; Bauscher and Mäntele, 1992; Buchanan *et al.*, 1992). The isotopic shifts in this spectral region and the assignments of the dominating semiquinone vibration are summarized in Table III. The band at 1486 cm^{-1} is shifted by 1- (Δv = −16 cm^{-1}) and 3-^{13}C (Δv = −8 cm^{-1}) labelling and is therefore dominated by the 1-C⸚O vibration. The difference spectra of the labelled compounds in which this band is shifted to 1478 (Figure 3b) and 1470 cm^{-1} (Figure 6b) indicate that its true size is masked by a negative band. The band at 1466 cm^{-1} is dominated by the 4-C⸚O vibration (Δv = −36 cm^{-1} by 4-^{13}C labelling), but has a large contribution from the 1-C⸚O vibration (Δv = −25 cm^{-1} by 1-^{13}C labelling) and the 1/2-C⸚C ring vibration (Δv = −21 cm^{-1} by 2-^{13}C labelling). The band at 1457 cm^{-1} seems not to be shifted by the quinone labels used. The band at 1447 cm^{-1} is equally shifted by labels at the 1, 2 and 4 positions (see Table III); it seems to represent a C⸚C ring vibration. Interestingly, the label at position 3 shows no isotopic effects on this band.

What can be learned from these assignments?

The equivalence of positions 1 and 4 and positions 2 and 3, as observed in free UQ$_{10}$, is disturbed by specific interactions with the protein environment, as indicated by the different isotopic shifts at the different positions. In

agreement, ENDOR measurements suggest that hydrogen bonding of the two ubiquinone C=O groups in the protein is not equivalent (Feher *et al.*, 1985).

Electrochemically induced difference spectra between the reduced and neutral states of UQ_0, UQ_1 and UQ_{10} have been measured (Bauscher *et al.*, 1990; Bauscher and Mäntele, 1992). The difference spectra of UQ_1 and UQ_{10} in general agree, and show almost no shifts of the C=O and the C=C frequencies in different solvents. Independent of the solvent polarity, comparing the frequencies of UQ_{10} in CH_2Cl_2 ($\varepsilon = 9$) and UQ_1 in MeCN ($\varepsilon = 38$), the C=O and C=C vibrations appear at 1650 and 1610 cm^{-1}, respectively (Bauscher and Mäntele, 1992). Also, the different capability to form hydrogen bonds in the different solvents does not induce bandshifts of the neutral UQ_1 in MeCN and MeOH (Bauscher and Mäntele, 1992). In contrast, the frequencies of the 1- and 4-C=O vibrations of UQ_{10} when bound to the protein are differently shifted. The 1-carbonyl vibration is seen at 1660 cm^{-1} in the protein, similar to free UQ_{10}. This indicates similar binding, as in solvents, and weak interactions with the protein environment. The 4-C=O vibration shows a dramatic downshift and dominates the band at 1601 cm^{-1}. Such downshift is not mimicked in the electrochemical studies and the question arises which specific interaction could cause such a large downshift indicating a decrease in bond order of the C=O group? Local point charges influencing the π-electron distribution are not located within a distance of 10 Å of the 4-C=O group, making a specific influence only at position 4 unlikely (Allen *et al.*, 1988; Ermler *et al.*, 1992). Furthermore, NMR experiments give no indication for local point charges in the microenvironment of the 4-C=O group (H.J.M.de Groot, personal communication). Alternatively, hydrogen bonding of the C=O group should in principle shift the frequency to lower wave numbers (Zadorozhnyi and Ishchenko, 1965). Steric interactions of UQ_{10} in the binding pocket, e.g. a distortion of the methoxy groups (Robinson and Kahn, 1990), could push negative partial charges to the oxygen of the 4-C=O group, thereby facilitating a strong hydrogen bond to the environment. Actually, in contrast to the electrochemical studies, infra-red measurements of quinhydrone complexes at which strong hydrogen bonding of the quinone carbonyls is established, show frequency shifts between 30 and 60 cm^{-1} (Slifkin and Walmsley, 1970). This arrangement seems to mimic, at least in part, the binding of the 4-C=O group to the protein. The larger shift observed in the protein (60 in contrast to 30 cm^{-1} in *p*-benzoquinone−hydroquinone complex) can be explained by a stronger hydrogen bond or by an additional interaction of the 4-C=O group with the protein. On the other hand, strong hydrogen bonding is not supported by solid-state NMR experiments (H.J.M.de Groot, personal communication); however, a fast changing hydrogen bond to different donors, for example, would not be resolved in the NMR and may explain the discrepancies. Nevertheless, further studies have to be performed to clarify which interaction reduces the bond order of the 4-C=O group. In particular, experiments at low temperature, at which the band of the 4-C=O group shows up in the NMR experiments, have to be performed to specify in more detail the strong interactions of the 4-C=O group with its environment.

Interestingly, the structures of the *Rb.sphaeroides* RCs

obtained by X-ray crystallography propose the two carbonyl oxygens of Q_A to be within hydrogen bonding distance of the protein, while the exact binding partners are unclear. One carbonyl group is reported to be hydrogen bonded to the peptide nitrogen of Ala M260 (Allen *et al.*, 1988; Chang *et al.*, 1991; Ermler *et al.*, 1992), while the other carbonyl group is forming a hydrogen bond to either the side chain of Thr M222 (Allen *et al.*, 1988; Chang *et al.*, 1991) or His M219 (Ermler *et al.*, 1992). A flipping hydrogen bond between the Thr and His could explain the different results of the X-ray studies. Because His M219 also seems to be bound to the Fe^{2+}, a 4-C=O (Q_A)−His M219−Fe^{2+}−His L190−C=O (Q_B) arrangement would represent an attractive fixed structural element (Allen *et al.*, 1988). Strong hydrogen bonding or a strong interaction of the 4-C=O group in such an arrangement could facilitate the electron transfer from Q_A to Q_B, and might contribute to the factors governing the different redox reactions of UQ_{10}. At the Q_B site, in contrast to the Q_A site, UQ_{10} is protonated after reduction.

The semiquinone C≕C and C≕O vibrations of UQ_1 and UQ_{10} show almost no frequency shifts in solvents with different polarity (CH_2Cl_2 and MeCN), but are shifted to lower wave numbers when the hydrogen bonding capability of the solvents is increased. The C≕O and C≕C vibrations of UQ_1 appear at 1486 cm^{-1} in MeCN, and are shifted due to hydrogen bonding in MeOH to 1466 (C≕O) and 1424 cm^{-1} (C≕C; Bauscher and Mäntele, 1992). Comparing these results with the frequencies of Q_A^- vibrations, the following conclusions are drawn. The mode dominated by the 1-C≕O vibration absorbs mainly at 1486 cm^{-1} as UQ_1 in MeCN and UQ_{10} in tetrahydrofuran, indicating weak interactions with the protein as already seen for the ground state. On the other hand, the 4-C≕O group contributes a dominant vibration to the band at 1466 cm^{-1}, similar to UQ_1 in MeOH, indicating a strong hydrogen bond to the protein.

In summary, in the ground state the 1-C=O group is weakly bound to the protein with similar interactions as in aprotic solvents. In contrast, reduced bond order is observed at the 4-C=O group and it appears to have a strong interaction with the protein. In the charge-separated state a strong hydrogen bond of the 4-C≕O group to the environment is found, whereas the 1-C≕O group remains more weakly hydrogen bonded.

Investigations of the factors determining the Q_A binding site show that the minimal requirements for occupancy of the Q_A site and electron transfer reactions via Q_A are one ring and a heteroatom (Warncke and Dutton, 1993a,b). Further investigations show, in good agreement with the results presented here, that the loss of one carbonyl oxygen atom does not influence significantly the strength of interaction with the site, and that a second carbonyl oxygen atom provides a binding free energy of −3.6 kcal/mol (Warncke and Dutton, 1993b). Zadorozhnyi and Ishchenko (1965) correlate empirically hydrogen bond energies with frequency shifts of the C=O vibration in different compounds for a C=O···HO hydrogen bond (Zadorozhnyi and Ishchenko, 1965). Even though their equation might not describe correctly the correlation in the protein, yielding therefore larger values (a frequency shift of −60 cm^{-1} would correspond to ∼−8 kcal/mol), the value of −3.6

kcal/mol seems too small to account fully for the frequency shift.

Very recently C=O and C=C vibrations of Q_A and Q_A^- in *Rb.sphaeroides* RCs have been assigned by Breton *et al.* (1994). In contrast to the approach presented here, they used ubiquinone uniformly ^{13}C-labelled or ^{18}O-labelled on both carbonyl oxygens. This allows the unspecific assignment of C=O/C=C ubiquinone vibrations. In very good agreement with the results presented here, they assigned the bands at 1660, 1628 and 1601 cm^{-1} to C=O/C=C vibrations and the bands at 1484, 1466 and 1447 cm^{-1} to C≕O/C≕C vibrations (compare Tables II and III).

However, specific assignments to single groups using such an approach are principally not possible. Only tentative assignments can be performed and here the situation is complicated due to the strong coupling between the C=O and C=C vibrations when UQ_{10} is bound at the protein. Nevertheless, their tentative assignments to single groups, and conclusively the proposals based thereon, have to be modified.

(i) The band at 1628 cm^{-1} was assigned to a C=O (tentatively to the 1-C=O) vibration, and the band at 1601 cm^{-1} was assigned to the C=C vibration, even though already strong C=C and C=O character to the respective bands has been recognized. In contrast, the site-specific labels used here indicate a dominant contribution of the 4-C=O group to the band at 1601 cm^{-1} and a dominant contribution of the 1-C=O group to the band at 1660 cm^{-1}. These assignments are strongly supported by the observation that the band at 1601 cm^{-1} is not shifted by the label at position 1, whereas the band at 1660 cm^{-1} is not shifted by the label at position 4. Based on these assignments, a strong binding of the 4-C=O group to the protein but not of the 1-C=O group is concluded.

(ii) The semiquinone bands at 1484, 1466 and 1447 cm^{-1} were assigned to C≕C, C≕O and C≕C vibrations, respectively, and symmetric binding of the 1- and 4-C≕O groups to the protein environment was proposed. In contrast, the specific labels indicate different frequencies for the 1 and 4 vibrations as given in Table III, which allow us to propose an asymmetric binding to the protein also in the reduced state.

Materials and methods

Synthesis of UQ_{10}, specifically ^{13}C-labelled at positions 1–4 respectively, was performed as described by van Liemt *et al.* (1994). RC protein was purified from *Rb.sphaeroides* strain R26 (Feher and Okamura, 1978). The native UQ_{10} cofactor was removed from the Q_A and Q_B sites and the Q_A site was reconstituted with native and the labelled UQ_{10}, respectively, as described by Okamura *et al.* (1975). Subsequent washing of the RCs on a DEAE column with 4% LDAO in 10 mM Tris, pH 8, for 1 h was performed to remove residual UQ_{10} at the Q_B site. Thereby, the Q_B site was depleted to >95%, as determined by photobleaching the primary donor at 865 nm and fitting the recombination kinetics to a sum of two exponentials: $A = A_1 \exp(-k_1 t_1) + A_2 \exp(-k_2 t_2)$. Upon normalization of the amplitudes A_1 and A_2 ($A_1 + A_2 = 100\%$), belonging to the fast Q_A^- and the slow Q_B^- decay, respectively, the fraction of RCs with functional secondary quinone was obtained.

The occupancy of the Q_A site was analysed by measuring the photobleaching at 865 nm under continuous illumination; 100% activity was determined by adding a 100-fold excess of UQ_{10} (3 mg/ml stock solution in 10 mM Tris, 1% Triton X-100) to a test sample. The activity of the used samples ranged from 84 to 90%. As shown in a control experiment, Q_A-depleted RCs provide a flat baseline in the infra-red spectral region under the experimental conditions described below.

$Q_A^- - Q_A$ FTIR difference spectra were recorded as described by Breton *et al.* (1991a). 45 µl 35 µM *Rb.sphaeroides* R26 RCs dissolved in 10 mM Tris buffer, 0.1% LDAO, 1 mM EDTA, pH 8, were deposited on a CaF_2 window and concentrated to ~350 µM under nitrogen. 5 µl 40 mM Tris buffer, pH 8, containing 100 µM terbutryn (to replace the left Q_B), 5 mM diaminodurene (DAD) and 2 mM sodium ascorbate (redox compounds) were added. After further careful drying to a volume of ~1 µl, the sample was covered with another CaF_2 window and thermostabilized at 10°C in the FTIR apparatus.

Infra-red spectra of the RCs were recorded on a Bruker IFS 88 instrument, equipped with an MCT detector (Heβling *et al.*, 1993). A total of 256 scans were averaged for each spectrum. The difference was taken between spectra recorded during steady state illumination with actinic light >700 nm and in darkness. About 200 difference spectra were co-added, resulting in a difference spectrum ultimately consisting of ~50 000 averages.

To visualize more clearly the bandshifts caused by isotopic labelling, double difference spectra between the $Q_A^- - Q_A$ spectra with labelled and unlabelled UQ_{10} were computed, minimizing the function Δ^2, with Δ = (difference spectrum with labelled UQ_{10}) $- a$ (difference spectrum with unlabelled UQ_{10}) $+ b$ (Gerwert and Siebert, 1986). To determine a and b, minimization was performed between 1800 and 1675 cm^{-1}; a spectral region where no bandshifts were observed. The obtained values for a and b were then used to compute the double difference spectra between 1800 and 1000 cm^{-1}.

Infra-red spectra of the ubiquinones were taken on a Bruker IFS 66v instrument. The ubiquinones were dissolved in *n*-pentane and deposited on a CaF_2 window. After evaporation of the pentane the remaining transparent ubiquinone film was measured in the infra-red. A total of 1024 scans was averaged for each spectrum. The ubiquinone spectra were baseline corrected using the Bruker instrument software.

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