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Binding mode of 1,4-naphthoquinones at the Q_A-site of the reaction centre from *Rhodobacter sphaeroides* investigated by light-induced FTIR difference spectroscopy

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Introduction

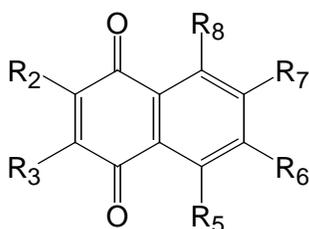
The reaction centre (RC) from the photosynthetic purple bacterium *Rhodobacter sphaeroides* (*R. sphaeroides*) is an integral membrane protein-pigment complex that catalyses the conversion of light energy into chemical energy. The primary steps of light-induced electron transfer include the rapid electron transfer from the primary excited donor D, a bacteriochlorophyll dimer, to the primary electron acceptor Q_A, a ubiquinone-10 (UQ-10) molecule, and further to the secondary ubiquinone Q_B.

Recently, methyl substituted 1,4-naphthoquinone compounds have gained substantial interest as artificial primary quinones since they bind tightly to the Q_A site of the reaction centre

enabling its functional reconstitution while retaining the native ubiquinone at Q_B (see Labahn *et al.* 1995, Graige *et al.* 1996).

Schmid *et al.* (1999) reported the synthesis of naphthoquinone derivatives containing different numbers of methyl groups in various positions of the ring system and, in some cases, an additional undecyl-tail in position 3 (Fig. 1).

In this work we have applied FTIR difference spectroscopy to investigate the binding mode of 6- or 7-methylated naphthoquinone compounds at the Q_A binding site. In the presence of an external reductant of the primary donor, the Q_A⁻/Q_A difference spectra exclusively show the absorbance changes of both the quinone and its environment in the RC upon reduction of the primary quinone. The spectra were interpreted in the light of results obtained for ubiquinone and vitamin K₁ (Breton *et al.* 1992, 1994a, 1994b, Brudler *et al.* 1994).



R₂, R₅-R₈ = H, CH₃
R₃ = H, CH₃, C₁₁H₂₃

Fig. 1. Nomenclature of 1,4-naphthoquinones.

Materials and methods

Quinones. 2,3,6-Trimethyl-1,4-naphthoquinone (236TMNQ), 2,6-dimethyl-3-undecyl-1,4-naphthoquinone (26DM3UNQ) and 2,7-dimethyl-3-undecyl-1,4-naphthoquinone (27DM3UNQ) were synthesized according to published procedures (Schmid *et al.* 1999). Vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) was purchased from Aldrich.

Reaction centres. Reaction centres were isolated from photosynthetically grown cells of the *Rhodobacter sphaeroides* strain *R-26* as described (Feher *et al.*, 1978). The ubiquinones bound at Q_A and Q_B were removed by incubating the RCs with 1,10-phenanthroline (Okamura *et al.*, 1975). The residual quinone content was less than 0.1 ubiquinone/RC.

Quinone reconstitution and FTIR sample preparation. Reconstitution of the Q_A -site with the naphthoquinone compounds was done by adding an ethanolic solution of the corresponding quinone ($\sim 70 \mu\text{M}$) to quinone-depleted RCs (120 nM) suspended in MKM-buffer (10 mM MOPS (3-[N-morpholino] propanesulfonic acid), 50 mM KCl, 0.04 % dodecyl- β -D-maltoside, pH 7.2). After equilibrating the system for at least 2 h at 20 °C, the reconstituted RCs were concentrated to a volume of 50 μl , using Microcon YM-100 centrifugal filter devices (Millipore Corp.). To avoid harmful salt and detergent concentrations, the solution was diluted with 450 μl MK-buffer containing no detergent and reduced salt concentration (1 mM MOPS, 5 mM KCl, pH 7.2) prior to a second concentration step, yielding a volume of 10 μl . This sample was deposited onto the depression of a special CaF_2 window. After addition of 5 μl of an aqueous solution of the redox mediator diaminodurene (DAD, 2.5 mM) and sodium ascorbate (1.25 mM) as reductant for the primary donor, the droplet was further concentrated under a smooth stream of nitrogen. Before complete dryness, the RC film was sealed with a second CaF_2 window, yielding the FTIR sample with a thickness of a few microns.

FTIR spectroscopy. Light-minus-dark Q_A^-/Q_A difference spectra were measured at 2 ± 0.2 °C on a Bruker IFS 66v/S spectrometer equipped with a MCT-detector. Interferograms were recorded before and during continuous illumination ($\lambda=590$ nm). Twenty illumination cycles, separated by a dark time of 7 minutes to ensure full relaxation of the RCs to the ground state were averaged, resulting in ~ 40000 interferograms/spectrum. The difference spectra were normalized with respect to the spectral regions between 1500 and 1560 cm^{-1} and between 1670 and 1750 cm^{-1} by applying the vector normalisation method. The absorption spectra of the quinones were measured in *n*-heptane (2 mM) at 20 °C.

Results

The absorption spectra of the 6- or 7-methylated naphthoquinone compounds are very similar to the spectrum of vitamin K_1 (Fig. 2) which has been characterised by Breton and coworkers (Breton *et al.*, 1994a). In the region of the CO stretch vibration and the CC ring vibrations (1700-1550 cm^{-1}), the absorption spectra of 236TMNQ, 26DM3UNQ and 27DM3UNQ are almost identical. Within the accuracy of the method, the positions of the CO modes and of the quinonic CC modes are identical with those of vitamin K_1 . However, a 6 cm^{-1} frequency upshift of the aromatic ring vibrational mode was found.

Breton *et al.* (1994a) showed that different regions in the Q_A^-/Q_A difference spectrum of RCs from *Rhodobacter sphaeroides* with vitamin K_1 as Q_A can be distinguished (Fig.3): In the range of 1750-1670 cm^{-1} and 1560-1500 cm^{-1} the spectrum is dominated by non-quinonic contributions resulting from the response of the RC upon Q_A reduction. Below 1500 cm^{-1} , the spectrum is indicative for the absorption bands of the semiquinone anion radical whereas the signals of vitamin K_1 in its neutral state are located between 1670 and 1560 cm^{-1} . They are superimposed with strong contributions of the protein and possibly of other cofactors. In the non-quinonic regions, the difference spectra of 6/7-methylated compounds show a high similarity in terms of the shape and the characteristic frequencies of the absorption bands compared to those of vitamin K_1 (Fig. 3). Only small differences in the intensities of the signals were observed.

At 1444 cm^{-1} a strong signal was found for all three compounds. Shapes and intensities of these absorption bands coincide with that of vitamin K_1 observed at the same frequency. The latter observation was assigned by Breton *et al.* (1994a) to a CO vibrational mode of the vitamin K_1 semiquinone anion radical. A close analogy between the bands peaking at $1480\text{--}1482\text{ cm}^{-1}$ and the signal at 1478 cm^{-1} of vitamin K_1 was observed. However, the two additional bands of the CC modes at $1394/1388\text{ cm}^{-1}$ in the vitamin K_1 spectrum are absent in those of the methyl substituted naphthoquinones. A signal of comparable intensity close to this frequency was found at 1372 cm^{-1} . Furthermore, as a clear distinction to the vitamin K_1 difference spectrum, the spectra of 236TMNQ, 26DM3UNQ and 27DM3UNQ show an additional peak at 1466 , 1468 and 1469 cm^{-1} , respectively.

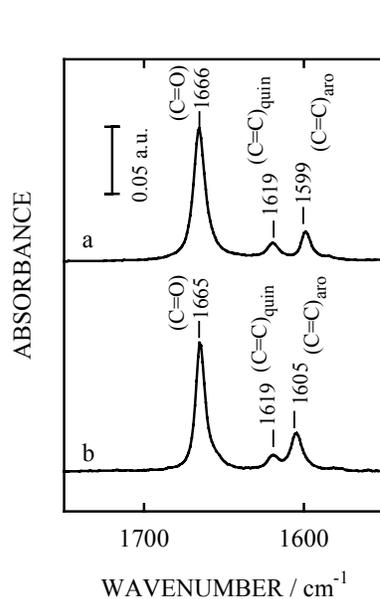


Fig. 2. Absorption spectra of (a) vitamin K_1 and (b) 26DM3UNQ in n-heptane. The carbonyl stretch vibrational mode, the quinonic and the aromatic ring vibrational modes are indicated as $(C=O)$, $(C=C)_{\text{quin}}$ and $(C=C)_{\text{aro}}$, respectively. a.u.: absorbance units. Accuracy of the frequency values: $\pm 1\text{ cm}^{-1}$.

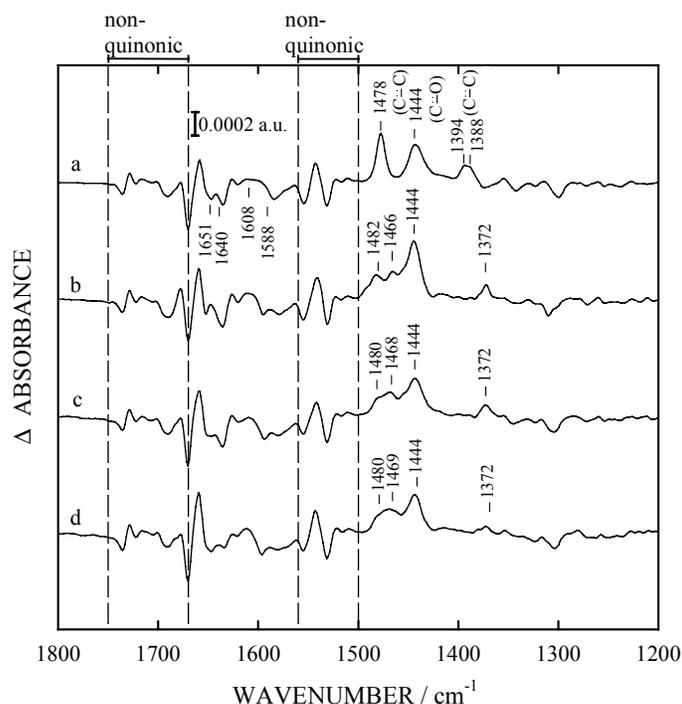


Fig. 3. Light-induced Q_A^-/Q_A -difference spectra of RCs from *Rhodospirillum rubrum* reconstituted with (a) vitamin K_1 , (b) 236TMNQ, (c) 26DM3UNQ, (d) 27DM3UNQ. Accuracy of the frequency values: $\pm 1\text{ cm}^{-1}$. Regions without spectral contribution of the quinones are indicated (designated 'non-quinonic'). The assigned modes of the vitamin K_1 radical anion are marked (Breton *et al.*, 1994a, see text for details).

Discussion

Differences in the non-quinonic regions of the Q_A^-/Q_A difference spectra of vitamin K_1 and the different naphthoquinones would indicate that quinone binding leads to structural changes of the Q_A binding site, depending on the structure of the corresponding quinone compound. The high similarity of these spectral regions in the difference spectra of 236TMNQ, 26DM3UNQ and 27DM3UNQ compared to that of vitamin K_1 (Fig. 3) therefore supports the assumption of a common binding mechanism for all functionally binding quinones to a rigid Q_A site. This, together with the good agreement of the corresponding CO stretch and the CC ring vibrations in the FTIR *absorption* spectra of the pure compounds

(Fig. 2), justifies the use of the vitamin K₁ difference spectrum as a basis for the interpretation of the difference spectra of the 6/7-methylated naphthoquinones: Based on these findings one would expect similar *in vivo* quinone signals for vitamin K₁ and the methylated naphthoquinones in the Q_A⁻/Q_A difference spectra as well.

The bands in the difference spectra peaking at 1444 cm⁻¹ and 1480-1482 cm⁻¹ coincide well with the signals of the vitamin K₁ difference spectrum (Fig. 3). Therefore, they are assigned to the CO mode and a CC mode of the semiquinone radical anion, respectively. In contrast, for the vibrations at 1466-1469 cm⁻¹ no equivalent was found in the vitamin K₁ spectrum. In case of ubiquinone, EPR/ENDOR measurements (Lubitz and Feher, 1999) and FTIR difference spectroscopy (Breton *et al.*, 1994a; Brudler *et al.*, 1994) revealed asymmetrical hydrogen bonds between the protein and the two carbonyl groups in the charge separated state D⁺Q_A⁻. Thus, the FTIR difference spectrum exhibited two distinct CO stretching modes for the semiquinone anion as opposed to vitamin K₁ where only one such mode was found. In the light of these results, the additional signal at 1466-1469 cm⁻¹ was attributed to a second CO vibrational mode. Based on this preliminary assignment we conclude that the methyl group in position 6 or 7 of the aromatic naphthoquinone ring (Fig. 1) breaks the symmetry of the two hydrogen bonds between the carbonyl groups of these quinones and the protein in the charge separated state D⁺Q_A⁻. This symmetry was found to be characteristic for vitamin K₁ and menaquinone K₉ (Breton *et al.* 1994a).

Work is in progress to verify the assignments by means of isotopically labelled naphthoquinone compounds. A detailed knowledge of the quinone spectral contributions is a prerequisite for using these naphthoquinones to elucidate the complex coupling mechanism of electron transfer, proton transfer and conformational changes in the RC protein.

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