Biochimica et Biophysica Acta 808 (1985) 464–469
Elsevier

BBA 40050

BBA Report

Electron nuclear double resonance of semiquinones in reaction centers of *Rhodopseudomonas sphaeroides*

W. Lubitz *, E.C. Abresch, R.J. Debus, R.A. Isaacson, M.Y. Okamura and G. Feher **

Department of Physics, B-019, University of California at San Diego, La Jolla, CA 92093 (U.S.A.)

(Received February 4th, 1985)
(Revised manuscript received April 15th, 1985)

Key words: Reaction center; ENDOR; Semiquinone; (*Rps. sphaeroides*)

Replacement of Fe^{2+} by Zn^{2+} in reaction centers of *Rhodopseudomonas sphaeroides* enabled us to perform ENDOR (electron nuclear double resonance) experiments on the anion radicals of the primary and secondary ubiquinone acceptors Q_A^- and Q_B^-. Differences between the Q_A and Q_B sites, hydrogen bonding to the oxygens, interactions with the protons of the proteins and some symmetry properties of the binding sites were deduced from an analysis of the ENDOR spectra.

In photosynthesis, light is converted into chemical energy by a charge-separation process followed by a series of electron-transfer reactions. To understand these processes quantitatively, a knowledge of the electronic structure of the reactants (donors and acceptors) is indispensable. The electron nuclear double resonance technique (ENDOR) has been used extensively, in particular in bacterial photosynthesis, to elucidate the electronic structure of the primary donor (a bacteriochlorophyll dimer) [1–4] but, so far, only scantily to explore the electron acceptors (two protein-bound quinones) [5]. The reason is that strong magnetic coupling of the quinones to a high spin Fe^{2+} broadens the EPR line [6] and makes ENDOR experiments difficult. Removal of Fe [7,8] facilitates the observation of ENDOR [5], but may change the structure of the reaction centers. We have developed a simple procedure to replace Fe^{2+} by the diamagnetic metal ion Zn^{2+} in reaction centers of *Rhodopseudomonas sphaeroides*. This reduces the EPR line width at 9 GHz by two orders of magnitude and makes ENDOR experiments feasible. We report here our preliminary ENDOR results on quinones in reaction centers in which Fe^{2+} was replaced by Zn^{2+}.

Reactions centers from *Rps. sphaeroides* R-26 were extracted with lauryldimethylamine *N*-oxide (LDAO) and purified as described [9]. Excess LDAO was removed by dialysis against 10 mM Tris-HCl (pH 7.7)/0.1% sodium cholate/0.1 mM EDTA. To dissociate the iron the dialysed mixture (reaction centers at $A_{1cm}^{800} = 10$) was incubated for 1 h in 1.5 M LiSCN/1 mM *o*-phenanthroline/10 mM Tris-HCl (pH 7.7)/0.03% sodium cholate. At the end of the incubation excess ubiquinone-10 (UQ-10) was added from a solution in 10% sodium deoxycholate and the iron was dialysed out. The Fe-free reaction centers were incubated overnight

* Present address: Institut für Organische Chemie, Freie Universität Berlin, 1000 Berlin 33, Germany.
** To whom correspondence and reprint requests should be addressed.

Abbreviations: ENDOR, electron nuclear double resonance; LDAO, lauryldimethylamine *N*-oxide; Cyt, cytochrome; DDQ, 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone; DQ, duroquinone, i.e., tetramethyl-1,4-benzoquinone; EtOH, ethanol; DME, 1,2-dimethoxyethane..

in 50 mM Tris-HCl (pH 7.7)/1 mM $ZnCl_2$; the excess Zn was removed subsequently by dialysis. All operations were performed in the dark at 4°C. The metal content of the final preparation was determined by atomic absorption to be approx. 0.1 Fe and approx. 1 Zn per reaction center.

The one-electron reduction of the primary quinone, Q_A, in reaction centers ($A_{1cm}^{800} = 25$) was accomplished either photochemically by illuminating the sample at room temperature in the presence of a 10-fold excess of Cyt c^{2+} or by chemical reduction with 50 mM dithionite/100 mM Tris-HCl. The secondary quinone, Q_B, was reduced photochemically by applying one short (approx. 0.4 μs) laser flash in the presence of Cyt c^{2+} [6]. Following reduction, the samples were quickly frozen by plunging them into liquid nitrogen. In some experiments the native quinone (UQ-10) was replaced by other quinones as previously described [10]. ENDOR spectra in frozen solutions (−150°C) were taken at 9 GHz with a set-up described earlier [4]. EPR experiments at 1 GHz were performed on a spectrometer that used a loop-gap cavity [11] followed by a circulator and low-noise preamplifier [6].

In general, one can distinguish three classes of protons responsible for the ENDOR spectrum of quinones in reaction centers. (I) Protons associated with the protein in the vicinity of the binding site (matrix ENDOR [12]); (II) exchangeable protons forming, for example, hydrogen bonds with the carbonyl oxygens of the quinone; and (III) non-exchangeable protons on the quinone, e.g., methyl, methoxy or methylene protons (see structure in inset of Fig. 1A). The ENDOR lines of Q_A^- in reaction centers are normally due to all three classes of protons (see Fig. 1A). To assign the ENDOR lines to a particular class of protons we used the technique of isotopic substitution. Fig. 1B shows the ENDOR spectrum obtained from Q_A^- in reaction centers that had been equilibrated with ^2H_2O for 2 days at 23°C. These lines are due to class I and III protons only. Fig. 1C shows the results obtained with reaction centers in which Q_A was replaced with deuterated (≥ 85%) UQ-10 extracted from bacteria grown in a medium containing ^2H_2O and deuterated succinate [13]. These ENDOR lines are due to class I and II only.

The five smallest splittings a–a' to e–e' had

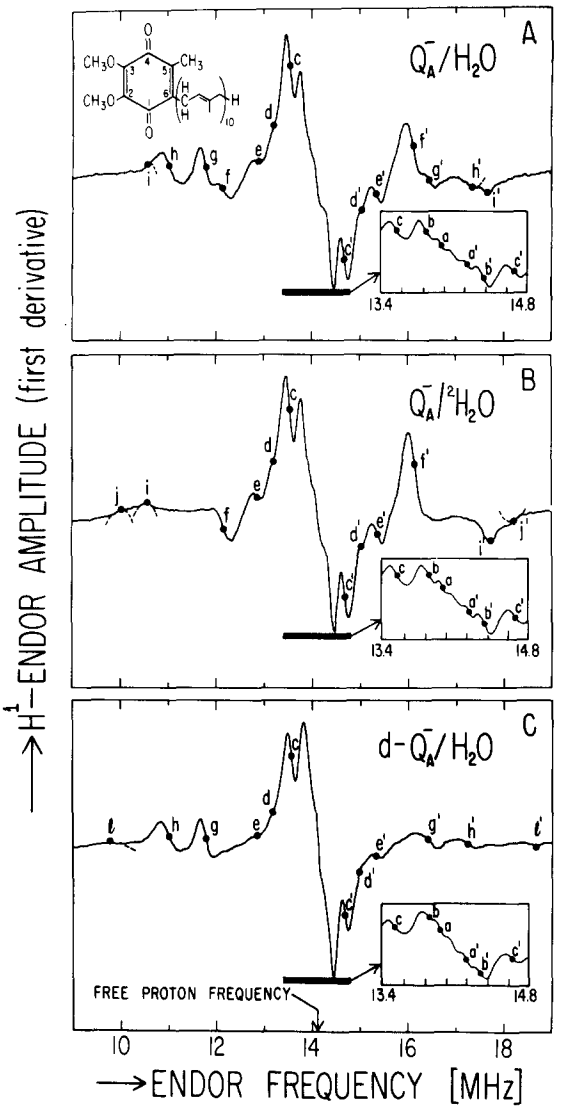

Fig. 1. ^{1}H-ENDOR spectra of Q_A^- (UQ-10) in reaction centers from *Rhodopseudomonas sphaeroides* R-26 from which the second quinone was removed [10]. (A) Reaction centers in their normal H_2O environment [9]. Top left inset shows structure of UQ-10. Bottom right inset shows expanded sweep of the central (matrix) ENDOR spectrum. (B) Reaction centers were dialyzed against ^2H_2O to remove exchangeable protons. (C) Reaction centers were depleted of their native UQ-10 and reconstituted with deuterated UQ-10. Experimental conditions: $T = -150$°C; $\nu_{elec} = 9.3$ GHz; microwave power, 2 mW; R.F. power, 100 W; frequency modulation, 200 kHz for full sweep and 100 kHz for expanded sweep (inserts). When the frequency modulation was lowered, additional lines were resolved (see heading in Table I). Magnetic field was set at center of EPR line. Sweep time, 4s/sweep. Typically ~1000 sweeps were averaged.

approximately the same value in all three samples (see insets of Fig. 1 and Table I). Most of them are due to non-exchangeable protons of the protein (class I).

ENDOR lines due to exchangeable protons (class II) were identified by comparing the spectra of Figs. 1A and 1B. When reaction centers were equilibrated with ^2H_2O, lines g–g' and h–h' were eliminated. They have, therefore, been assigned to exchangeable protons. In accord with this assignment, lines g–g' and h–h' occur prominently in reaction centers with deuterated quinones in H_2O (Fig. 1c). The exchangeable protons are most likely forming hydrogen bonds with the two carbonyl oxygens of the quinones. To obtain the total hf coupling of the exchangeable protons, we compared the EPR linewidths of the ^2H_2O exchanged sample with the unexchanged one. To eliminate g-anisotropy broadening [8] we measured the line widths at 1.2 GHz. The line narrowed from 7.5 gauss (peak-to-peak of the derivative signal) to 6.0 gauss in the deuterium-exchanged sample. This corresponds to a contribution of $(7.5^2 - 6.0^2)^{1/2} = 4.5$ G (i.e., approx. 13 MHz) to the observed line width. However, the two lines g–g' and h–h' can contribute at most $(4.7^2 + 6.5^2)^{1/2} = 8$ MHz to the width. Consequently, there must be other ENDOR lines (besides g–g' and h–h') associated with the exchangeable protons. Line l–l' in Fig. 1C is a likely candidate; its amplitude could be enhanced by setting the external magnetic field off the center of the EPR line (data not shown). This is a known procedure for selecting hyperfine coupling components in radicals having an anisotropic g value [12]. Thus, line l–l' may represent the anisotropic hyperfine coupling component $A_{\|}$, of either line g–g' or h–h'.

Of the protons associated with the quinone molecule, the methyl protons are expected to give a strong ENDOR signal [14,15]. At −150°C the protons are freely rotating, giving rise to an approximately axial hfc tensor. We assign the most prominent line in Fig. 1B, lines f–f' (4.0 MHz) and i–i' (7.1 MHz), to the two components, $A_{\perp}$ and $A_{\|}$, of the methyl hyperfine coupling tensor. The anisotropy is unusually large for a rotating methyl group, but is in approximate agreement with values found on isolated quinones [15]. Calculations show that the large anisotropy is mainly due to the vicinity of the oxygen. The isotropic hyperfine coupling $A_{iso} = \frac{1}{3}(2A_{\perp} + A_{\|})$ is 5.0 MHz, which has to be compared with the values of 5.7 and 5.9 MHz found by Das et al. in solutions [16] (see Table II). The discrepancy between these values can be explained by invoking a redistribution

TABLE I

^{1}H-ENDOR TRANSITIONS OF Q_A^- IN REACTION CENTERS FROM *RPS. SPHAEROIDES* R-26

QU, ubiquinone-10. UQ/^2H_2O, reaction centers equilibrated in ^2H_2O. d-UQ, deuterated ubiquinone. For experimental conditions, see legend to Fig. 1. The accuracy with which the transition frequencies were determined was in most cases governed by the unknown line shape. It is ~ ±0.02 MHz for hyperfine coupling components <1 MHz and ~ ±0.1 MHz for hyperfine coupling components >2 MHz. When the frequency modulation was lowered from 100 kHz to 40 kHz, an additional line at 0.09 MHz was observed and line b–b' showed a partially resolved structure at 0.46 and 0.57 MHz. In view of the smaller signal amplitude at 40 kHz, the higher frequency modulations (100 kHz and 200 kHz) were routinely used.

Transition	Hyperfine couplings (MHz)	Tentative Assignment
Sample: UQ/H_2O (Fig. 1A)		
a–a'	0.26	matrix ENDOR
b–b'	0.56	matric ENDOR
c–c'	1.13	matrix ENDOR
d–d'	1.8	matrix ENDOR
e–e'	2.6	matrix ENDOR
f–f'	4.0	$CH_3(A_{\perp})$
g–g'	4.7	exchangeable ^{1}H
h–h'	6.5	exchangeable ^{1}H
i–i'	7.1	$CH_3(A_{\|})$
Sample: UQ/^2H_2O (Fig. 1B)		
a–a'	0.26	matrix ENDOR
b–b'	0.53	matrix ENDOR
c–c'	1.14	matrix ENDOR
d–d'	1.9	matrix ENDOR
e–e'	2.6	matrix ENDOR
f–f'	4.0	$CH_3(A_{\perp})$
i–i'	7.1	$CH_3(A_{\|})$
j–j'	8.0	?
Sample: d-UQ/H_2O (Fig. 1C)		
a–a'	0.26	matrix ENDOR
b–b'	0.51	matrix ENDOR
c–c'	1.13	matrix ENDOR
d–d'	1.9	matrix ENDOR
e–e'	2.5	matrix ENDOR
g–g'	4.7	exchangeable $^1H(A_{\perp})$
h–h'	6.4	exchangeable ^{1}H
l–l'	9.0	exchangeable $^1H(A_{\|})$

TABLE II

HYPERFINE COUPLINGS (IN MHz) OF METHYL PROTONS OF DIFFERENT QUINONES INCORPORATED IN REACTION CENTERS FROM *RPS. SPHAEROIDES* R-26

For experimental conditions see legend to Fig. 1. The accuracy of the transition frequencies is $\sim \pm 0.1$ MHz (see footnote in Table I). An axial hyperfine coupling component tensor is assumed, i.e., $A_{iso} = \frac{1}{3}(2A_{\perp} + A_{\parallel})$.

Environment	$A_{\perp}$	$A_{\parallel}$	A_{iso}	Reference
Quinone: DQ				
Q_A in reaction center	3.3	6.0	4.2	this work
	5.2	8.5	6.3	this work
	6.0	9.3	7.1	this work
Frozen EtOH	4.4	7.5	5.4	15
EtOH			5.3	16
Quinone: DDQ				
Q_A in reaction center	4.0	7.1	5.0	this work
	5.6	9.1	6.8	this work
Quinone: UQ-10				
Q_A in reaction center	4.0	7.1	5.0	this work
EtOH			5.7	16
DME			5.9	16
Q_B in reaction center	4.7	7.9	5.8	this work

of the electron density in UQ-10 possibly due to hydrogen bonding.

The redistribution of the electron density was further investigated in reaction centers in which the native UQ-10 was replaced by either 2,3-dimethoxy-5,6-dimethyl (DDQ) or tetramethyl (DQ) benzoquinone. In DDQ we observed two sets of methyl hyperfine splittings (Table II). One had the same value as in UQ-10 (5.0 MHz). The second splitting is larger (6.8 MHz), presumably due to preferential hydrogen bonding to one of the oxygens. The inequivalence of the two methyl groups is similar in origin to the one observed in the presence of hydrogen bonding or counterions in solutions [17]. The average of the two hyperfine coupling constant in DDQ is 5.9 MHz which is in good agreement with the value found by Das et al. for UQ-10 in solution (see Table II). The fact that we see only two sets of methyl lines (rather than four for a binding site that lacks 2-fold symmetry around the 1–4 molecular axis of the quinone; see below) suggests that DDQ binds to the protein in a unique orientation. The matrix ENDOR lines observed in DDQ had the same value as in UQ-10. This shows that both quinones, UQ-10 and DDQ, bind to the same site.

The ENDOR spectrum of DQ shows three sets of methyl splittings (see Table II) indicating that the binding site is asymmetric with respect to the 1–4 molecular axis of the quinone. Assuming that the smallest splitting is doubly degenerate, the average of the four isotropic hyperfine couplings is 5.4 MHz, in excellent agreement with the values found by O'Malley and Babcock for DQ in frozen ethanol [15] and by Das et al. in solution [16] (see Table II). The matrix ENDOR lines are shifted from those found in UQ-10 indicating a displacement of the quinone ring with respect to the UQ-10 binding site.

The hyperfine couplings of the two methylene protons of the isoprenoid chain in UQ-10 have so far not been identified. A detailed comparison of the spectra obtained from UQ-10 in ^2H_2O and DDQ in ^2H_2O should facilitate the identification (unpublished data). Similarly, the protons of the methoxy group have not been identified. These are expected to have small hyperfine couplings [16,18], most likely in the region of the matrix ENDOR.

We now turn to the important question of the difference between the electronic structure of Q_A^- and the secondary acceptor, Q_B^-. This difference must be due to the surrounding protein, which

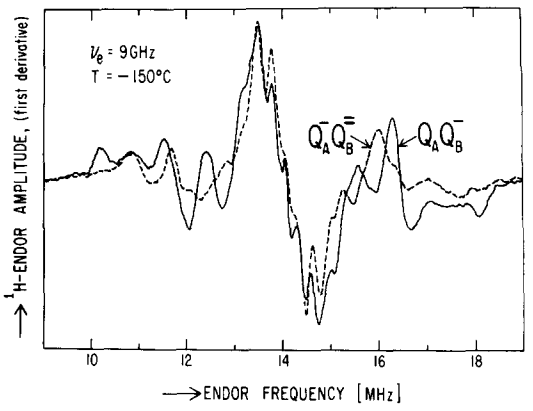

Fig. 2. Comparison of ^{1}H-ENDOR spectra of $Q_AQ_B^-$ (full line) and $Q_A^-Q_B^{2-}$ (dashed line) in reaction centers from *Rps. sphaeroides* R-26. The $Q_A^-Q_B^{2-}$ spectrum is the same as obtained from Q_A^- in reaction centers having one quinone (see Fig. 1A). The $Q_AQ_B^-$ spectrum has an admixture of Q_A^- (see text). Experimental conditions the same as described in Fig. 1.

causes a reduction in the redox potential of Q_A^- [19]. Reaction centers in the $Q_AQ_B^-$ state were prepared by using a short intense laser flash [6]. However, after the laser flash approx. 30–50% of the reaction centers still remained in the Q_A^- state. Thus, the ENDOR spectrum (Fig. 2) corresponded to a mixture of Q_A^- and Q_B^-, which made the analysis more difficult. Our preliminary findings were as follows. The isotropic hyperfine coupling of the methyl group is 16% larger than in Q_A^- (see Table II) indicating an increase in spin density at position 5 (see structure in inset of Fig. 1A) and a predicted concomitant decrease at position 6. This tends to equalize the hfc's at positions 5 and 6. Such an effect could arise, for example, from a weakening of the hydrogen bond to one oxygen. This explanation is supported by the fact that the hyperfine coupling of the exchangeable protons that form the hydrogen bonds were found to be smaller in Q_B (3.1 and 5.0 MHz). This could, perhaps, account for the weaker binding of Q_B to the reaction center protein [10].

Besides ^{1}H-ENDOR we also looked for ^{15}N-ENDOR from Q_A^- in isotopically substituted Zn reactions centers. The ^{15}N-spectrum revealed lines in the vicinity of ^{15}N Larmor frequency, indicating the presence of a nitrogen in the surroundings of UQ-10. This is consistent with an imidazole nitrogen of a histidine being near the quinone binding site as postulated by Hearst and Sauer [20]. We also determined the hyperfine couplings with ^{17}O from the EPR spectrum (at 2.1 K) of Zn reaction centers reconstituted with isotopically substituted DQ. The outermost hyperfine lines ($m_I \approx \pm 5/2$) were split indicating the inequivalence of the two carbonyl oxygens on the quinone. The total splittings of the two $\pm 5/2$ lines were 143 and 166 G corresponding to hyperfine couplings of 80 and 93 MHz, respectively. DQ frozen in ethanol (2.1 K) showed unsplit lines corresponding to a hyperfine coupling of 82 MHz. These splittings are much larger than those found in model compounds at room temperature. The reason is that at low temperatures the anisotropic hyperfine interaction is not averaged out, and, therefore, contributes to the observed splitting.

In summary, the EPR/ENDOR studies have so far provided the following information. The spectra of Q_A^- and Q_B^- indicate interactions with residues from the surrounding protein; further isotope substitution experiments may help identify the residues. Both Q_A^- and Q_B^- interact with exchangeable hydrogens, which, as proposed by Hales [21] form bonds to the carbonyl oxygen(s). The environment of Q_A^- is asymmetric as shown from the diffence in the hyperfine coupling with the two ^{17}O, the non-equivalent methyl hyperfine couplings in the symmetric DQ^- and DDQ^- and the deviation of the methyl hyperfine couplings in $(UQ\text{-}10)^-$ from that found in isotropic media. The asymmetry may be due to different strengths of the H-bonding to the oxygens. Another possible cause is the presence of a positive charge, possibly Zn^{2+}. Such a charge may stabilize Q^- in a hydrophobic environment and may play a role in the electron-transfer process. The hyperfine coupling constants of Q_A^- and Q_B^- are different. These differences are presumably related to the change in redox potential that leads to the vectorial electron transport from Q_A^- to Q_B. Further ENDOR studies should contribute to our understanding of the structure and function of quinones in reaction centers as well as in other photosynthetic and bioenergetic systems [22].

We thank G.T. Babcock for a stimulating discussion on model compounds and P.L. Loach for a gift of DDQ. This work was supported by grants from the National Institute of Health (GM 13191) and the National Science Foundation (PCM 82-02811). W.L. held a research fellowship from the Max Kade Foundation (New York).

Note added in proof (Received June 13th, 1985)

At -20°C we have observed in reaction centers containing 0.75 Q per reaction center two sets of transitions, corresponding to hyperfine coupling constants of 5.1 and 8.4 MHz. We have tentatively assigned these to $A_\perp$ and $A_\parallel$ of one of the methylene protons. We have also determined the ^{17}O hyperfine couplings in Q_A^- and Q_B^- in reaction centers reconstituted with isotopically enriched UQ-10. For Q_A^- the hyperfine couplings were 75 and 94 MHz. For Q_B^- only one set of lines, corresponding to a hyperfine coupling of 85 MHz, was observed.

References

1 Norris, J.R., Scheer, H. and Katz, J.J. (1975) Ann. N.Y. Acad. Sci. 244, 260–280

2 Feher, G., Hoff, A.J., Isaacson, R.A. and Ackerson, L.C. (1975) Ann. N.Y. Acad. Sci. 244, 239–259

3 Lubitz, W., Lendzian, F., Scheer, H., Gottstein, J., Plato, M. and Möbius, K. (1984) Proc. Natl. Acad. Sci. USA 81, 1401–1405

4 Lubitz, W., Isaacson, R.A., Abresch, E.C. and Feher, G. (1984) Proc. Natl. Acad. Sci. USA 81, 7792–7796

5 Okamura, M.Y., Debus, R.J., Isaacson, R.A. and Feher, G. (1980) Fed. Proc. 39, 1802 (Abstr. No. 1037)

6 Butler, W.F., Calvo, R., Fredkin, D.R., Isaacson, R.A., Okamura, M.Y. and Feher, G. (1984) Biophysical J. 45, 947–973

7 Loach, P.A. and Hall, R.L. (1972) Proc. Natl. Acad. Sci. USA 69, 786–790

8 Feher, G., Okamura, M.Y. and McElroy, J.D. (1972) Biochim. Biophys. Acta 267, 222–226

9 Feher, G. and Okamura, M.Y. (1978) The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 349–386, Plenum Press, New York

10 Okamura, M.Y., Isaacson, R.A. and Feher, G. (1975) Proc. Natl. Acad. Sci. 72, 3491–3495

11 Froncisz, W. and Hyde, J.S. (1982) J. Mag. Res. 47, 515–521

12 Kevan, L. and Kispert, L.D. (1976) in Electron Spin Double Resonance Spectroscopy, p. 239, J. Wiley, New York

13 Dougherty, R.C., Crespi, H.L., Strain, H.H. and Katz, J.J. (1966) J. Am. Chem. Soc. 88, 2854 and Katz, J.J., Dougherty, R.C., Crespi, H.L. and Strain, H.H. (1966) J. Am. Chem. Soc. 88, 2856–2857

14 Hyde, J.S., Rist, G.H. and Eriksson, L.E.G. (1968) J. Phys. Chem. 72, 4269–4276

15 O'Malley, R.J. and Babcock, G.T. (1984) J. Chem. Phys. 80, 3912–3913

16 Das, M.R., Connor, H.D., Leniart, D.S. and Freed, J.H. (1970) J. Am. Chem. Soc. 92, 2258–2268

17 Oakes, J. and Symons, M.C.R. (1970) Trans. Faraday Soc. 66, 10–17

18 Rabold, G.B., Ogata, R.T., Okamura, M.Y., Piette, L.H. Moore, R.E. and Scheuer, P.J. (1967) J. Chem. Phys. 46, 1161–1167

19 Wraight, C.A. (1982) in Function of Quinones in Energy Conserving Systems (Trumpower, B.L., ed.), pp. 181–197, Academic Press, New York

20 Hearst, J.E. and Sauer, K. (1984) Z. Naturforsch. 39c, 421–424

21 Hales, B.J. and Case, E.E. (1981) Biochim. Biophys. Acta 637, 291–302

22 Trumpower, B.L. (ed.) (1982) Function of Quinones in Energy Conserving Systems, Academic Press, New York