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INTERACTION OF EXOGENOUS BENZOQUINONE WITH PHOTOSYSTEM II IN CHLOROPLASTS

THE SEMIQUINONE FORM ACTS AS A DICHLOROPHENYLDIMETHYLUREA-INSENSITIVE SECONDARY ACCEPTOR

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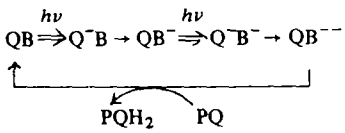
Chloroplasts were submitted to a sequence of saturating short flashes and then rapidly mixed with dichlorophenyldimethylurea (DCMU). The amount of singly reduced secondary acceptor (B^-) present was estimated from the DCMU-induced increase in fluorescence in the dark caused by the reaction: $QB^- \xrightarrow{DCMU} Q^-B$. By varying the time interval between the preillumination and the mixing, the time course of B^- reoxidation by externally added benzoquinone was investigated. It was found that benzoquinone oxidizes B^- in a bimolecular reaction, and does not interact directly with Q^-. When a sufficient delay after the preillumination was allowed in order to let benzoquinone reoxidize B^- before the injection of DCMU, the fluorescence increase caused by one subsequent flash fired in the presence of DCMU was followed by a fast decay phase ($t_{1/2} \simeq 100$ μs). The amplitude of this phase was proportional to the amount of B^- produced by the preillumination. This fast decay was observed only after the first flash in the presence of DCMU. These results are interpreted by assuming a binding of the singly reduced benzoquinone to Photosystem II where it acts as an efficient, DCMU-insensitive, secondary (exogenous) acceptor.

Introduction

In plant PS II, as well as in bacterial reaction centers, the link between the 'primary' acceptor (one-electron carrier) and the pool of quinones (two-equivalent carriers) involves a special quinone able to store one electron until another light reaction provides a second electron, triggering a transfer of two equivalents to the pool [1–4].

In chloroplasts, the occurrence of such a mechanism with a secondary acceptor called B (or R) was revealed from flash sequence experiments showing binary oscillations either in the amount of electrons transferred to PS I [2], or in the dark increase in the chlorophyll fluorescence yield induced by adding the PS II inhibitor DCMU following illumination [1]. Velthuys [1] explained the latter result by assuming that DCMU somehow modifies the equilibrium constant between Q and B so that the centers which are in the (quenching) state QB^- before DCMU addition are converted into the Q^-B (fluorescent) state.

The oscillatory phenomena observed are in agreement with the scheme:



where PQ is a plastoquinone of the pool and PQH_2 its doubly reduced state (the protonation steps have been omitted for simplicity). In dark-adapated iso-

Abbreviations: DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem.

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lated chloroplasts, the majority of centers are in the QB state.

Wollman [5] has shown that externally added *p*-benzoquinone is able to oxidize B^- which is otherwise stable in the dark (particularly in intact algae). In a recent paper [6], we reported results obtained by continuously monitoring the fluorescence after rapid mixing of flash-preilluminated chloroplasts with DCMU. This enabled us to confirm the occurrence of binary oscillations for the peak of the DCMU-induced transient of fluorescence. By varying the time interval between a preilluminating flash and the DCMU mixing, the time course of B^- reoxidation by benzoquinone can be followed.

In the present paper, we report results which suggest that singly reduced benzoquinone remains bound to the center where it can accept a second electron in a DCMU-insensitive reaction.

Material and Methods

Broken spinach chloroplasts (thylakoids) were prepared as described previously [6]. They were used in a medium containing 0.4 M sucrose, 50 mM Hepes, pH 8, and 10 mM $MgCl_2$.

Commercially available *p*-benzoquinone was recrystallized through sublimation and used in fresh solutions.

The experimental set-up was the same that as described in Ref. 6, except for the use of a second flash (F_2). A sample of chloroplasts was submitted to a sequence of saturating short flashes (F_1), then mixed by flowing through a mixing chamber, with an equal volume of reactant (suspending medium + 10^{-4} M DCMU). After this 300 ms flow period, the sample stopped in an observation cell facing the photomultiplier window. The fluorescence yield of chlorophyll was continuously monitored by synchronous detection of the fluorescence excited by a weak, modulated (1 kHz) beam from green light-emitting diodes. A second flash (F_2), from a flash-pumped dye laser (pulse duration 1 μs), provided saturating illumination within the observation chamber. No attempt to protect the photomultiplier was made, but an analog gate was used to ground the lock-in amplifier input during the light pulse in order to avoid the slow recovery following overloading. The blindness time after the flash was 15 ms.

For the experiment of Fig. 6 the set-up was modified to allow the monitoring of fast transients of the fluorescence yield after a flash (F_2). A gating system was used to protect the photomultiplier during the actinic flash, and a train of 256 detecting flashes (2-μs duration) was synchronized with the address advance of a multichannel analyzer. The intensity of these flashes was adjusted so that the whole train caused a negligible actinic effect. 5–10 accumulations were necessary to obtain a satisfactory signal-to-noise ratio. In order to eliminate a luminescence artifact, a subsaturating flash F_2 was used (hitting about half the centers), and the recording obtained in the absence of the detecting flashes was substracted from the overall signal.

Results

Fig. 1 shows the kinetics of B^- reoxidation after one flash in chloroplasts incubated with benzoquinone at two concentrations. The amount of B^- is revealed by the peak of the fluorescence transient upon mixing with DCMU at time Δt after the flash. All the experiments we report here were run at pH 8: at this pH the DCMU-induced increase in fluorescence

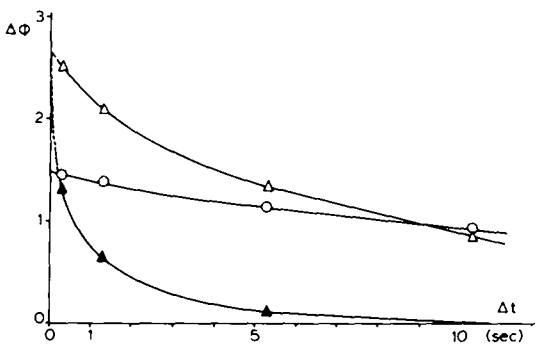

Fig. 1. Time course of B^- reoxidation in darkness. The peak of the DCMU-induced transient of fluorescence (arbitrary units) is plotted against the time Δt between one single preillumination flash and the mixing of the chloroplasts sample with DCMU. (o———o) Control without benzoquinone. The other curves were obtained after a 5 min dark incubation of the chloroplasts with $5 \cdot 10^{-5}$ (Δ———Δ) and $5 \cdot 10^{-4}$ M (▲———▲) benzoquinone. The dashed part is an arbitrary extrapolation for the 0.3 s flow period during which mixing is achieved. Due to some quenching of fluorescence at $5 \cdot 10^{-4}$ M benzoquinone, the starting value of the lower curve should be somewhat lower than indicated.

is fast compared to the decay (recombination) occurring afterwards, so that the peak of the transient is well defined. At lower pH values, the DCMU-induced increase is slower [6] and accordingly some truncation of the peak occurs. However, we checked that the phenomena described in this paper remain qualitatively valid at pH 6.

Two effects of benzoquinone on B^- are observed. Even at the low concentration (50 μM), it increases the initial amount of B^- after one flash, due to the oxidation of QB^- centers present in dark-adapted chloroplasts. Upon increasing the concentration to 500 μM, the reoxidation of B^- becomes faster. Concurrently, some quenching of fluorescence takes place. Due to this complication and to the nonlinear relationship between the fluorescence level and the amount of Q^-, no detailed kinetic analysis was attempted. However, it was checked that the rate of QB^- reoxidation by benzoquinone varies roughly as the product of chlorophyll and benzoquinone concentrations, suggesting a bimolecular reaction. No acceleration of the fluorescence decay was observed (not shown) in the presence of benzoquinone, either for the DCMU-induced transient after a preillumination flash, or for the flash-induced transient in the presence of DCMU. The oxidized benzoquinone thus interacts specifically with B^-, not with Q^-.

Fig. 2 (lower curves) is a plot of the peak of the DCMU-induced fluorescence transient in a flash sequence experiment (time interval between flashes 0.2 s, mixing with DCMU immediately after the last flash, except for curve c'). At 10^{-5} M benzoquinone (curve b), the main effect compared to the control (curve a) is a lowering of the zero-flash level (and (not shown) of the levels following even numbers of flashes) and an increase in the one-flash level (and (not shown) of the levels following odd numbers of flashes), consistently with the oxidation of dark-adapted QB^- centers, whereas the downward shift of curve c ($5 \cdot 10^{-4}$ M benzoquinone) is probably due to fluorescence quenching by the quinone. Curve c' was obtained at the same benzoquinone concentration as curve c, but with a 5 s delay before DCMU addition so that B^- had been mostly reoxidized.

We shall now be concerned with the fluorescence increase elicited by flash F_2 when it is fired 1 s after DCMU mixing (i.e., soon after the peak of the

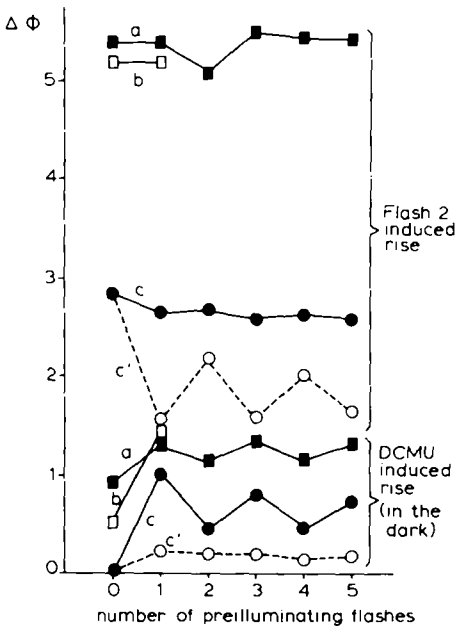

Fig. 2. Fluorescence levels after DCMU mixing in flash sequence experiments at various benzoquinone concentrations. Lower curves: peak of the DCMU-induced fluorescence transient (measured 0.5 s after the onset of mixing). Upper curves: fluorescence levels 15 ms after flash F_2. The procedure is: n preillumination flashes F_1 spaced 0.2 s apart, mixing with DCMU immediately after the last flash, except for curves c' where the flow period starts 5 s after the last F_1; flash F_2 is fired 1 s after the end of the flow period. Curves a, no benzoquinone; b, 10^{-5} M benzoquinone; c and c', $5 \cdot 10^{-4}$ M benzoquinone. Chloroplasts were incubated with benzoquinone, when present, for 5 min in the dark. Only the first points were plotted in curves b for the sake of clarity. The fluorescence levels are given in arbitrary units (but on the same scale for all data). The origin of the vertical scale was taken arbitrarily at the first point of curves c and c'. The dark-adapted level (ϕ_0) of a sample with no addition would be close to the first point of curve b.

DCMU-induced fluorescence transient). The upper curves in Fig. 2 show the results of such an experiment. The fluorescence yield was measured 15 ms after the flash, a time which is normally not critical due to the slow decay of fluorescence in the presence of DCMU ($t_{1/2}$ 1–2 s). The control sample (curve a) does not yield a flat sequence but an oscillating pattern (periodicity of 4, minima after two (or six, not shown) preillumination flashes) as already reported by Etienne [7]. The effect of a high concentration ($5 \cdot 10^{-4}$ M) of benzoquinone is mostly an overall

quenching (curve c). However, if one allows the reoxidation of B^- by benzoquinone to take place during a 5 s delay between the last preillumination flash and the DCMU addition (curve c'), the F_2-induced fluorescence level displays marked binary oscillations, with maxima on even numbers of preillumination flashes (phase opposite to that of the DCMU-induced rise), and with the usual damping observed in the periodic phenomena driven by PS II. By varying the time between preillumination and mixing, a good correlation was found between the amount of B^- reoxidized and the extent of the 'quenching' observed upon firing F_2 (not shown).

One might account for the results of (upper) curve c' by assuming that reduced benzoquinone acts as a quencher: when enough time is allowed to reduce benzoquinone from B^- before DCMU addition, a low fluorescence yield is observed upon firing F_2. It seems unlikely, however, that this quenching would be due to direct bimolecular interaction of reduced benzoquinone with chlorophyll, for quinones are known as quenchers of chlorophyll fluorescence in their oxidized rather than reduced state. This point was confirmed by experiments aimed at accumulating reduced benzoquinone by allowing a 5 s spacing between, e.g., two preillumination flashes and again a 5 s delay before the mixing: the result (not shown) was a 'high' fluorescence level (close to that of upper curve c' for two flashes). The accumulation of reduced benzoquinone by successive flashes thus does not cause an increase in the quenching.

Fig. 3 shows the recovery curve for this effect of

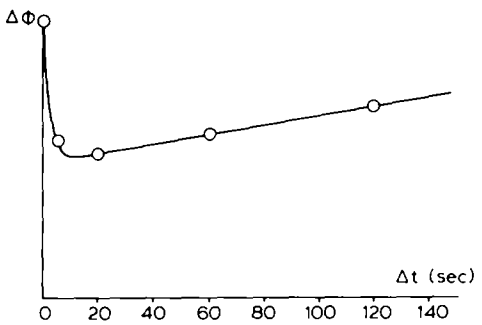

Fig. 3. Time course of the quenching effect due to singly reduced benzoquinone. The fluorescence level 15 ms after flash F_2 is plotted against the time interval Δt between one preillumination flash and the mixing with DCMU. F_2 is fired 1 s after the end of the mixing.

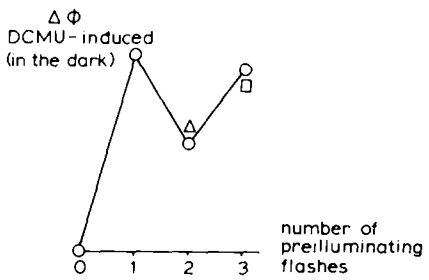

Fig. 4. Peak of the DCMU-induced fluorescence transient after 0–3 preillumination flashes in the presence of $5 \cdot 10^{-4}$ M benzoquinone. (○) Flashes F_1 spaced 0.2 s apart, mixing immediately after the last flash. (△) Same, except for a 5 s interval between the first and second flash. (□) 5 s interval between the first and second flash, 0.2 s interval between the second and the third flash.

benzoquinone. It is a plot of the fluorescence level measured 15 ms after F_2 as a function of the time interval Δt between one preillumination flash and the mixing with DCMU. The decreasing phase, as mentioned above, correlates well with the kinetics of B^- reoxidation by benzoquinone. It is followed by a slow rise (recovery) with $t_{1/2} \simeq 125$ s.

The experiment of Fig. 4 was designed to test the effect of B^- reoxidation by benzoquinone under conditions which do not involve F_2, but only the DCMU-induced fluorescence rise in darkness. A concentration of $5 \cdot 10^{-4}$ M benzoquinone was used throughout. The control (open circles) shows the usual pattern obtained for a short interval between

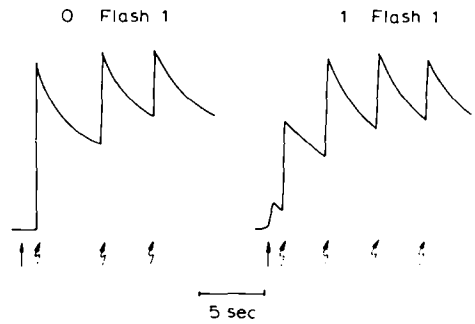

Fig. 5. Recordings of the fluorescence yield during a sequence of flashes F_2. No or one preillumination flash followed by a 5 s delay before mixing with DCMU. The straight arrow shows the time when the sample stops in the observation chamber after the mixing. The zig-zag arrows indicate the times when F_2 is fired.

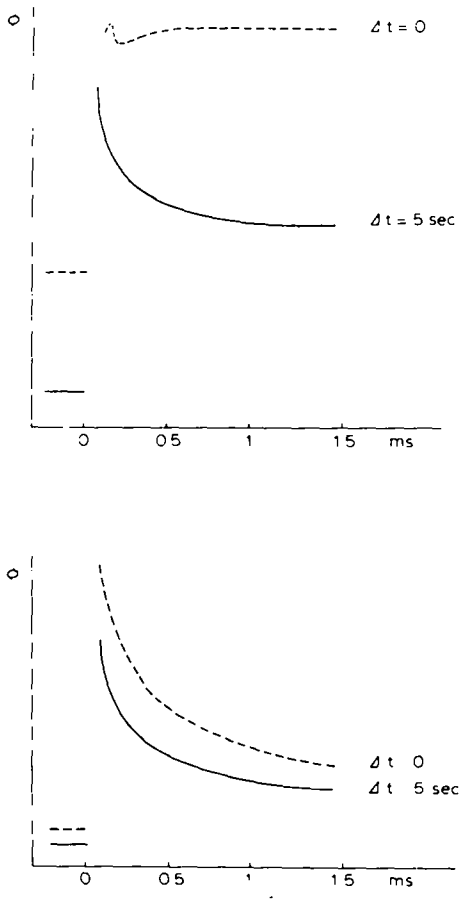

Fig. 6. Fast transient of the fluorescence yield elicited by flash F_2. Chloroplasts incubated with $5 \cdot 10^{-4}$ M benzoquinone are preilluminated by one flash, followed, immediately, or after a 5 s delay as indicated by the 0.3 s flow period which brings the sample into the observation chamber. Flash F_2 is fired 1 s after the sample has stopped, at time zero on the figure. Top: mixing with DCMU during the flow period. Bottom: no DCMU in the mixing medium. As described in Materials and Methods, the flash F_2 used in this experiment was attenuated to a subsaturating intensity (hitting about half of the centers) in order to minimize a luminescence artefact. The kinetics are the average of 10 accumulations, with subtraction of the artefact monitored under the same conditions but in the absence of analytical flashes.

the flashes F_1 with DCMU mixing immediately after the last flash. The triangle and square symbols show the results obtained after, respectively, two or three preillumination flashes, with a time of spacing of 5 s between the first and second flash. It appears clearly that this 5 s interval, although sufficient to allow B^- reoxidation, does not cause a phase shift of the oscillation. It thus turns out that the reoxidation of B^- by benzoquinone does not bring about an erasement of the system (binary) 'memory'.

We want now to investigate further the nature of the 'quenching' observed after an odd number of flashes F_1 in the experiment of Fig. 2, curve c'. Fig. 5 shows the effect of several flashes F_2 fired after no or one preillumination flash, followed by a 5 s delay in the presence of benzoquinone before the mixing with DCMU. In the zero flash F_1 experiment, a high fluorescence level was attained after the first flash F_2 and subsequent flashes did not elicit a noticeably higher level. On the other hand, after one preillumination flash, a low level was observed after the first F_2, whereas the subsequent flashes yielded essentially the high fluorescence level obtained in the zero flash F_1 experiment. In other words, the 'quenching' observed after an odd number of preillumination flashes is destroyed by one subsequent flash. This photochemical effect suggests the possibility that the low level of the fluorescence yield measured 15 ms after F_2 might be due to a fast reoxidation of Q^- undetected in the procedure we have used as yet. Accordingly, we ran experiments with a set-up allowing the detection of fast fluorescence transients (see Material and Methods). Fig. 6 (top) shows that there is indeed a fast decay of the fluorescence yield after F_2, with a $t_{1/2}$ of about 100 μs. This decay phase is observed only when benzoquinone was allowed to reoxidize B^- before the mixing with DCMU. It is absent when the mixing takes place immediately after the preillumination. Similarly, it is absent when no preillumination flash was given (not shown). The bottom part of Fig. 6 shows that this fast transient is observed as well in the absence of DCMU. The reoxidation of Q^- is then normally fast ($t_{1/2} \simeq 400$–600 μs [8–10]), and a definite acceleration of the decay is seen when benzoquinone has been allowed to reoxidize B^-.

Discussion

The following model provides a satisfactory explanation for the phenomena that have been described. The basic assumption is that singly reduced benzoquinone (benzosemiquinone) remains bound to the center until it accepts a second electron from Q^- (in a reaction that is insensitive to the DCMU block).

Schematically:

$$S_iQB \overset{h\nu}{\Rightarrow} S_{i+1}Q^-B \rightarrow S_{i+1}QB^- \xrightarrow[(1)]{bq} S_{i+1}QB \quad (bq^-)$$

$$\overset{h\nu}{\Rightarrow} S_{i+2}Q^-B \xrightarrow[(2)]{bqH_2} S_{i+2}QB \quad (bq^-)$$

where only the relevant steps are indicated. S_i is the donor system at oxidation state i, bq, bq^- and bqH_2 represent the oxidized benzoquinone, and its singly and doubly reduced forms, respectively. Step 1 is the reoxidation of B^- described in Fig. 1. Its rate depends on the benzoquinone concentration. Step 2 accounts for the fast fluorescence decay shown in Fig. 6 that occurs after an odd number or preillumination flashes when step 1 has been allowed to take place. This reaction, with $t_{1/2} \simeq 100$ μs, is about 5-times faster than the normal reoxidation of Q^- by B in the absence of DCMU. Remarkably, it is not blocked by DCMU.

The model accounts for the conservation of the binary 'memory' of the PS II acceptor side in spite of B^- reoxidation by benzoquinone, as observed in Figs. 2 and 4. This is due to the fact that the bound benzoquinone conserves the electron-storing properties of the endogenous plastoquinone B which it replaces as a secondary acceptor. This functional replacement of B does not take place only with DCMU present. This can be seen from Fig. 6 (bottom) which shows an acceleration of Q^- reoxidation in the presence of bound benzoquinone; this appears also in Fig. 4 which shows that the reoxidation of B^- by benzoquinone during the 5 s interval between the first and second flash does not change the phase of the DCMU-induced increase in fluorescence yield: therefore, after the second flash, the electron from Q^- is not stabilized on B, but diverted towards benzosemiquinone.

Within this explanatory framework, the slow recovery kinetics ($t_{1/2} \simeq 125$ s) seen in Fig. 3 correspond to the release of benzosemiquinone from the center.

The data we have reported in the paper suggest striking differences between the oxidized and singly reduced forms of benzoquinone with respect to their interaction with PS II. The oxidized benzoquinone reacts slowly with the center in a bimolecular process. Its target is B^- (however, an alternative possibility is envisaged below), and it is not able to reoxidize Q^- in the presence of DCMU. In the singly reduced state, however, it remains bound to the center for several minutes and accepts at a fast rate electrons from Q^-, whether or not DCMU is present. We believe that the ability to mimic with an externally added reagent the charge accumulation occurring on the acceptor side of PS II may be of interest for a better understanding of this process. Actually, it has been recently proposed by Velthuys [11] that the endogenous plastoquinone B has a very different affinity for its binding site on PS II when it is in its semiquinone form (strong binding to PS II) or in its totally oxidized or reduced forms which could exchange rapidly with free plastoquinone diffusing in the membrane.

It is interesting to compare further our results with those reported by Velthuys [11] in the same paper, studying the interaction of DBMIB with PS II. The author finds that DBMIB inhibits the rapid PQH_2 formation upon a second flash, provided that a sufficient time interval (in the seconds range) between the first and second flashes is allowed. The following mechanism is proposed (where D represents DBMIB):

$$QB \overset{h\nu}{\Rightarrow} Q^-B \rightleftharpoons QB^-$$

$$\downarrow D$$

$$D^-$$

$$Q^-D \rightleftharpoons QD^- \overset{h\nu}{\Rightarrow} Q^-D^- \rightarrow QDH_2$$

The formation of QDH_2 from Q^-D^- occurs with a half-time of about 30 ms, and DH_2 is subsequently released from the center. The main difference between this scheme and ours is that the exogenous oxidized quinone is assumed to interact with Q^- and not with B^-, although no experimental evidence is given about this particular point. The rationale for this reaction scheme arises from Velthuys' hypothesis of a single site on PS II for the binding of B or inhibitors such as antimyxin, DBMIB, or DCMU. In our scheme we were led to assume that oxidized benzoquinone accepted electrons from QB^-, and not from Q^-B, because of the low equilibrium $K = [Q^-B]/[QB^-]$, and, on the other hand, because no reoxidation of Q^- by benzoquinone could be observed in the presence of DCMU. However, the first argument is

not conclusive: the measured rate of B^- reoxidation by benzoquinone may result from a fast rate of the Q^- reoxidation by benzoquinone divided by the equilibrium constant K. The second argument would not hold either if one follows Velthuys' hypothesis of a unique site on Q for quinone or DCMU binding: when this site is occupied by DCMU, benzoquinone would be unable to reoxidize Q^-. Further work is thus needed to decide whether benzoquinone and/or DBMIB accept an electron from Q^- or B^-. It turns out anyway that exogenous quinones such as benzoquinone or DBMIB share several properties with the endogenous plastoquinone:

they remain bound to the center in their semiquinone form, whereas no such binding occurs with the oxidized and doubly reduced forms;

they accept a second electron form Q^- (with $t_{1/2}$ of, respectively, 100 μs, 500 μs and 30 ms for benzoquinone, plastoquinone and DBMIB).

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