

# Interactions of Halogenated Benzoquinones with the Non-Heme Iron (Q<sub>400</sub>) in Photosystem II

Hiroyuki Koike, Yasuhiro Kashino, and Kazuhiko Satoh

Department of Life Science, Faculty of Science, Himeji Institute of Technology (HIT), Harima Science Park City, Hyogo 678-12, Japan

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Interactions of halogenated benzoquinones with the acceptor side of Photosystem (PS) II were studied by measuring fluorescence induction curves and flash-induced absorbance changes in PS II particles isolated from *Synechococcus vulcanus*. Following results were obtained: 1) Addition of some halogenated benzoquinones prior to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the dark increased the area above the fluorescence induction curve (work integral) by a factor of two. 2) Based on the ability to increase the fluorescence work integral, halogenated benzoquinones could be divided into two groups. 3) 2,6-Dichlorobenzoquinone (2,6-DCBQ), trichlorobenzoquinone (TCBQ) and tetrahalogenated benzoquinones except tetrafluorobenzoquinone (fluoranyl) (group A) increased the work integral, but 2,5-DCBQ and fluoranyl (group B) did not. 4) Rapid reoxidation of Q<sub>A</sub><sup>-</sup> was observed in the presence of quinones which belong to group A.

These results were interpreted in terms of dark oxidation of Q<sub>400</sub> by quinones belonging to group A. Possible mechanisms of oxidation of Q<sub>400</sub> by these quinones in the dark are discussed.

## Introduction

On the reducing side of photosystem II, there are two bound plastoquinone molecules functioning as Q<sub>A</sub> and Q<sub>B</sub>. By absorption of light, an electron from P<sub>680</sub>, reaction center chlorophyll of PS II, is transferred to Q<sub>A</sub> via pheophytin *a*. The electron located on Q<sub>A</sub> is then transferred to Q<sub>B</sub> which acts as a two-electron gate: Q<sub>B</sub>-semiquinone is known to be very stable and quinol formed by the second electron from Q<sub>A</sub><sup>-</sup> is then supposed to be replaced by another plastoquinone molecule after protonation.

Presence of another redox component between Q<sub>A</sub> and Q<sub>B</sub> was first reported by Ikegami and Katoh [1]. They showed that the area above the fluorescence induction curve in the presence of DCMU (work integral) was increased by incubation of spinach chloroplasts with ferricyanide for a long time before addition of DCMU. This ferri-

cyanide effect was eliminated when DCMU was added before addition of ferricyanide [1, 2]. Because it has a relatively high redox potential (E<sub>m</sub> ≈ 400 mV) [1–3], this extra redox component was named Q<sub>400</sub> and has recently been identified as iron (II) by EPR and Mössbauer spectra [4]. It is also found that Q<sub>400</sub> is oxidized by semiquinone forms of some exogenous benzoquinone derivatives which are bound to the Q<sub>B</sub> site: An EPR signal of oxidized Q<sub>400</sub> appears after every odd numbered flashes [5] or after 273 K dark incubation following illumination at 200 K [6]. It should be noted that no benzoquinone derivative has been reported to oxidize Q<sub>400</sub> without being reduced to semiquinone.

In the present paper, we show that some halogenated benzoquinones as well as their semiquinones can oxidize Q<sub>400</sub> in the dark using PS II particles isolated from a thermophilic cyanobacterium, *Synechococcus vulcanus*. Compared with the case of ferricyanide, oxidation of Q<sub>400</sub> in the dark by these benzoquinones took place very rapidly (within 2 sec). Possible mechanisms for Q<sub>400</sub> oxidation in the dark are discussed.

## Materials and Methods

PS II particles from *S. vulcanus* thylakoids were prepared with lauryldimethylamine-N-oxide as described in ref. 7.

**Abbreviations:** PS II, Photosystem II; EPR, electron paramagnetic resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCBQ, dichloro-*p*-benzoquinone; TCBQ, trichloro-*p*-benzoquinone; chloranil, tetrachloro-*p*-benzoquinone; fluoranil, tetrafluoro-*p*-benzoquinone; bromanil, tetrabromo-*p*-benzoquinone; iodanil, tetraiodo-*p*-benzoquinone.

Reprint requests to H. Koike.

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The reaction mixture contained 1 M sucrose/50 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH (pH 6.0)/10 mM NaCl and 5 mM  $MgCl_2$  and PS II particles equivalent to 3.5  $\mu g$  chlorophyll/ml. Excitation light for fluorescence measurements was passed through a 5 cm layer of water, a Hoya HA-50 and a Corning 4-96 filter. Intensities of fluorescence  $> 650$  nm were monitored, and areas above the fluorescence induction curves (work integral) were calculated.

Flash-induced absorbance changes of  $Q_A$  were measured at 413.5 nm by a single beam spectrophotometer as described in ref. 8. The samples suspended in the same medium as for fluorescence measurements were excited by a xenon flash lamp (5  $\mu s$  duration) and 100 signals were averaged and analyzed.

Iodanil was a kind gift from Dr. W. Oettmeier, Ruhr University, Bochum. TCBQ was purchased from Aldrich Co. Other quinones were commercial products of Tokyo Kasei Co., Japan.

## Results

Fig. 1 shows effects of chloranil on a fluorescence induction curve. Areas above the curves normalized by intensities of variable fluorescence

(work integral) indicate the pool sizes of electron acceptors before the DCMU-inhibition site. When DCMU is added prior to addition of chloranil, the work integral "A" in Fig. 1 left can be regarded as to reflect  $Q_A$  reduction and then it corresponds to one electron per PS II [9]. It should be noted that the maximum level of fluorescence was decreased compared with that in the absence of chloranil (data not shown) due to non-photochemical quenching by the quinone [10] but normalized area "A" was almost the same. When chloranil was added before addition of DCMU, the work integral was increased twice as indicated "B" in Fig. 1 right (see also Table I). The ratio of the work integral, B/A, thus reflects the ability of benzoquinones to increase the pool size of electrons before the DCMU-inhibition site in the dark. Because further addition of ferricyanide along with chloranil did not increase the B/A ratio (data not shown) and its redox potential is high enough [11], we suppose that this increase was due to oxidation of  $Q_{400}$  by chloranil in the dark.

Table I summarizes the effects of halogenated benzoquinones on the ratio of the work integral, B/A. The work integral was not affected by 2,5-DCBQ, indicating no oxidation of  $Q_{400}$  in the dark, which is compatible with previous EPR

Table I. Effects of various quinones on the fluorescence work integral. Experimental conditions were as in Fig. 1. Work integral A and B were calculated by measuring the area as shown in the Fig. 1.

|     | 2,5-DCBQ<br>(50 $\mu M$ ) | 2,6-DCBQ<br>(50 $\mu M$ ) | TCBQ<br>(50 $\mu M$ ) | Chloranil<br>(2 $\mu M$ ) | Fluoranil<br>(50 $\mu M$ ) | Bromanil<br>(1 $\mu M$ ) | Iodanil<br>(1 $\mu M$ ) |
|-----|---------------------------|---------------------------|-----------------------|---------------------------|----------------------------|--------------------------|-------------------------|
| B/A | 0.99                      | 2.20                      | 2.32                  | 1.88                      | 1.02                       | 1.84                     | 2.18                    |

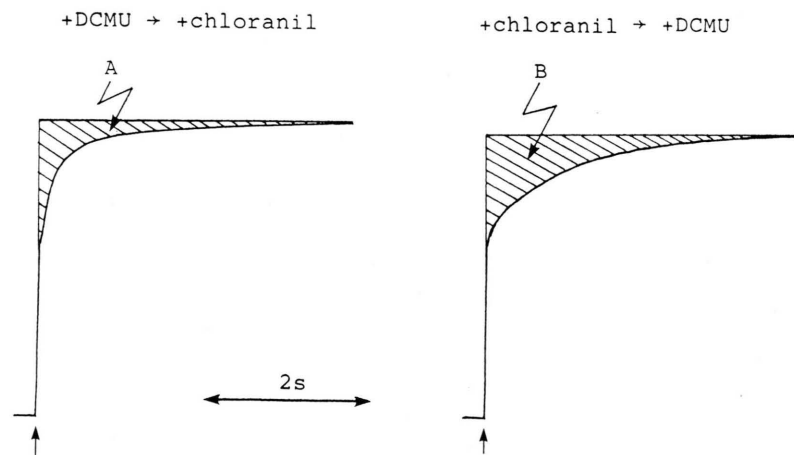


Fig. 1. Fluorescence induction curves in the presence of chloranil and DCMU: The effect of order of addition. Left: Fluorescence induction curve of PS II particles incubated with 5  $\mu M$  DCMU for 10 sec followed by addition of 2  $\mu M$  chloranil. Right: Fluorescence induction curve incubated with chloranil followed by addition of DCMU.

measurements [6]. It should be noted that the semiquinone form of 2,5-DCBQ, created by one flash excitation or continuous illumination at 200 K followed by annealing at 273 K in the dark oxidized  $Q_{400}$  to some extent [5, 6]. On the other hand, 2,6-DCBQ doubled the work integral, showing that 2,6-DCBQ can oxidize  $Q_{400}$  in the dark without being reduced to a semiquinone form. TCBQ also increased the work integral twice in the magnitude as well as chloranil. As in the case of chloranil, other two tetrahalogenated benzoquinones, bromanil and iodanil, also doubled the work integral, but surprisingly fluoranil did not.

If some halogenated benzoquinones oxidize  $Q_{400}$  in the dark, the next question is "how fast is  $Q_{400}$  oxidized by these quinones?" In order to measure the rate of dark oxidation, PS II particles were preincubated for various periods with halogenated benzoquinones before addition of DCMU (see inset of Fig. 2), the fluorescence induction curves were measured and work integrals were plotted. All the halogenated benzoquinones which increased the work integral close to two with 10 sec preincubation (Table I) doubled the work integral with all the preincubation time tested (5 sec ~ 2 min, Fig. 2). These results indicate that some

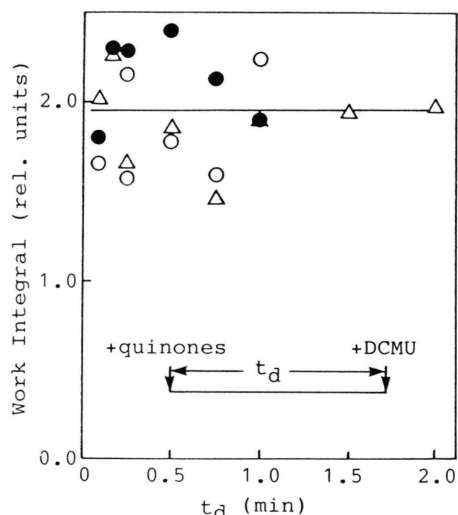


Fig. 2. Effects of dark incubation time with tetrahalogenated benzoquinones prior to addition of DCMU on the fluorescence work integral. The samples were preincubated with halogenated benzoquinones followed by addition of sufficient amount of DCMU to block electron transport from  $Q_A^-$  to  $Q_B$ . Open circles, bromanil; solid circles, iodanil; triangles, chloranil.

halogenated benzoquinones oxidize  $Q_{400}$  in the dark within 5 sec, the shortest incubation time to perform these experiments due to technical reasons.

It has also been reported that when  $Q_{400}$  is in an oxidized state, rapid electron flow from  $Q_A^-$  to  $Q_{400}$  occurs [6, 12]. In order to check if there is a rapid  $Q_A^-$  oxidation in the presence of these halogenated benzoquinones we measured flash-induced redox changes of  $Q_A$ . When  $Q_A$  is reduced by a flash, an absorbance increase in a blue region takes place due to semiquinone formation and due to an electrochromic shift of pheophytin caused by the semiquinone ( $Q_A^-$ ) [13, 14]. The following absorbance decrease after flash excitation represents the relaxation of  $Q_A^-$ . When  $Q_{400}$  is in a reduced state, the increased absorbance at 413.5 nm decays with a half time of 1–2 ms due to electron transfer from  $Q_A^-$  to  $Q_B$  in *Synechococcus* PS II particles [8, 15].

Fig. 3 shows the effects of various chloro-substituted benzoquinones on flash-induced absorbance changes at 413.5 nm. In the presence of 2,5-DCBQ, the absorbance increase was followed by relatively slow decay with a half time of 1.3 ms as reported previously [8]. This decay time corresponds to electron transfer from  $Q_A^-$  to  $Q_B$  suggesting that  $Q_{400}$  is not oxidized by 2,5-DCBQ during flash interval of 2 sec under present experimental conditions. This is compatible with the data listed in Table I and with those of Petrouleas and Diner that 2,5-DCBQ is a poor oxidant of  $Q_{400}$  even in its semiquinone form [6]. When 2,6-DCBQ was added instead of 2,5-DCBQ, rapid reoxidation of  $Q_A^-$  with a half time of 0.2–0.3 ms was observed. We suppose that this rapid reoxidation of  $Q_A^-$  corresponds to fast electron transfer from  $Q_A^-$  to  $Q_{400}^+$  as reported in ref. 6. Quite similar rapid reoxidation of  $Q_A^-$  was observed with TCBQ or chloranil (Fig. 3, lower curves). It should be noted that the traces in Fig. 3 are averaged time courses of 100 signals with flashes repeated at 0.5 Hz. If only semiquinone forms can oxidize  $Q_{400}$  as reported in ref. 5 and 6, rapid oxidation of  $Q_A^-$  by  $Q_{400}^+$  must take place every two flashes. This should result in a composite decay kinetics of 0.2–0.3 ms and 1–2 ms with an equal amplitude. However, this was not the case; more than 80% of the absorbance decay was attributed to the very fast decay component in all three cases (2,6-DCBQ, TCBQ and chloranil). These results suggest that all the

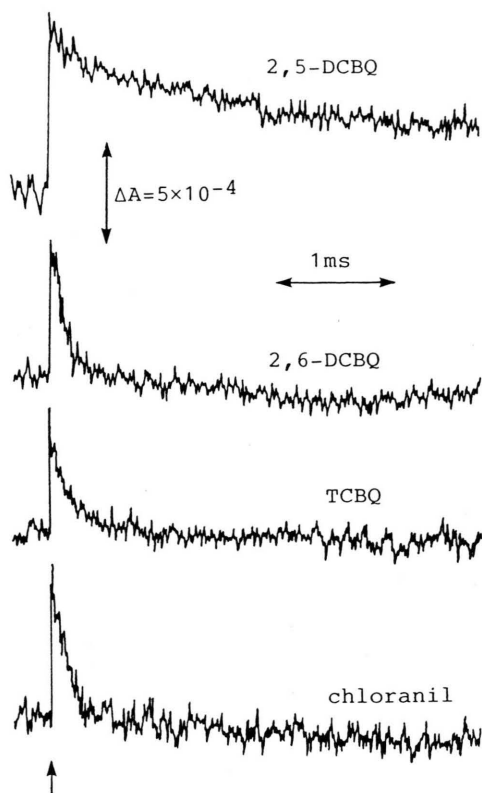
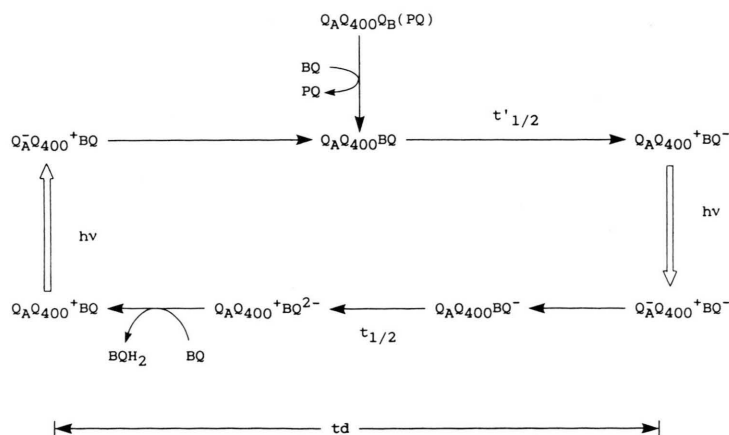


Fig. 3. Effects of halogenated benzoquinones on flash-induced absorbance changes at 413.5 nm. The samples were excited with xenon flashes at 0.5 Hz and 100 signals were averaged. Signals were measured in the presence of 0.4 mM 2,5-DCBQ, 0.4 mM 2,6-DCBQ, 50  $\mu$ M TCBQ plus 0.1 mM ferricyanide or 10  $\mu$ M chloranil plus 0.1 mM ferricyanide, respectively.

three quinones oxidize  $Q_{400}$  both in an oxidized forms and in semiquinone forms within a flash interval of 2 sec (see Scheme 1). This is in quite contrast to the case of methyl- or phenyl-substituted benzoquinones which oxidize  $Q_{400}$  only in a semiquinone state [6].

Although iodanil and bromanil also accelerated the decay of  $Q_A^-$ , fluoranil did not accelerate reoxidation kinetics of  $Q_A^-$ , indicating that  $Q_{400}$  is not oxidized rapidly either by an oxidized form nor a semiquinone form of fluoranil (data not shown). These results also agree with the idea that tetra-halogenated benzoquinones, except fluoranil, oxidize  $Q_{400}$  in the dark (Table I).

In order to investigate the rate of oxidation of  $Q_{400}$  by some halogenated benzoquinones in a time range shorter than 5 sec, we took advantage of the rapid reoxidation of  $Q_A^-$  by  $Q_{400}^+$ . We measured changes in the decay kinetics after flash-induced absorbance increases at 413.5 nm by varying flash intervals: If oxidation rates of  $Q_{400}$  by halogenated benzoquinones or benzosemiquinones ( $t_{1/2}$  or  $t'_{1/2}$  or Scheme 1) are fast enough,  $Q_{400}$  should be in an oxidized state before every flash excitation. If, however, the rate is slow compared to the flash interval, the fraction of reduced  $Q_{400}$  in PS II reaction center might increase, which will result in an increased percentage of the slow component (1–2 ms) in  $Q_A^-$  reoxidation kinetics. Thus, the oxidation rate of  $Q_{400}$  by halogenated benzoquinones or by benzosemiquinones in the dark can be estimated by varying the flash interval and by plotting



Scheme 1. A possible oxidation reduction cycle of halogenated benzoquinones bound to  $Q_B$  site during flash illumination.



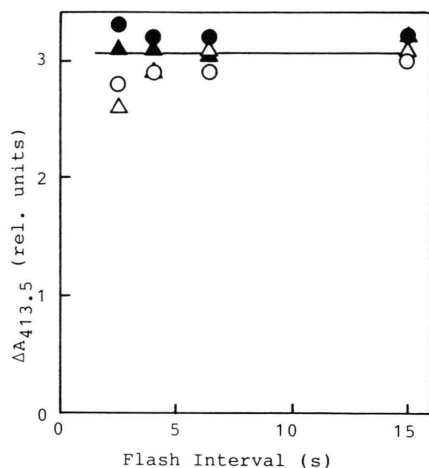


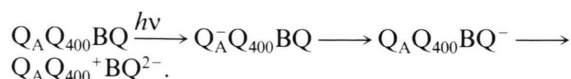
Fig. 4. Effects of flash intervals on the extent of the rapid decay component at 413.5 nm. Experimental conditions were as in Fig. 3. Open circles, 2,6-DCBQ; open triangles, TCBQ; solid triangles, chloranil. As a reference, extents of initial absorbance changes which show 1–2 ms decay in the presence of 2,5-DCBQ are also plotted (solid circles).

the ratio of the fast decaying component as a function of the flash interval.

As illustrated in Fig. 4, all the chloro-substituted benzoquinones tested showed no significant change in the magnitude of the fast decay component with flash intervals ranging from 2 to 15 sec. Time courses of absorbance changes in the presence of 2,5-DCBQ which does not oxidize  $Q_{400}$  were not altered by the increase in the flash interval as well. It is thus indicated that flash intervals tested in this experiment were long enough for relaxation of the acceptor side of PS II. These results suggest that  $Q_{400}$  was fully oxidized within 2 sec by both oxidized and semiquinone forms of these halogenated benzoquinones.

## Discussion

It has been reported that  $Q_{400}^+$  EPR signal is observed after every odd numbered flash [5] or after continuous illumination at 200 K followed by 0 °C annealing in the dark [6] in the presence of some exogenous benzoquinones. These phenomena are interpreted as a reductant-induced oxidation of  $Q_{400}$  by semiquinones of benzoquinone derivatives bound to the  $Q_B$  site [5] as shown below



In the present work, we found that  $Q_{400}$  could be oxidized just by adding some halogenated benzoquinones in the complete darkness. Furthermore, the rate of the  $Q_{400}$  oxidation was shown to be very rapid; it took place within the flash interval of 2 sec. Their semiquinones were also suggested to oxidize  $Q_{400}$  within 2 sec. This is in sharp contrast with the previous observation that only semiquinone forms of *p*-benzoquinone, methyl- or phenylbenzoquinones can oxidize  $Q_{400}$  [5, 6].

We failed to observe dark oxidation of  $Q_{400}$  by fluoranil and 2,5-DCBQ. This might be due to 1) fairly low binding affinities to the  $Q_B$  site, 2) insufficient redox potentials to oxidize  $Q_{400}$ , or 3) differences in the sitting position at the  $Q_B$  site. We can exclude the first possibility because affinities of 2,6-DCBQ and fluoranil to the  $Q_B$  site are higher than those of methyl-substituted benzoquinones whose semiquinone forms oxidize  $Q_{400}$  (to be published elsewhere). As to second possibility, Petrouleas and Diner [6] and Zimmermann and Rutherford [5] tried to explain the ability of quinones to oxidize  $Q_{400}$  in terms of the redox potentials of  $Q^-/QH_2$ . However, some halogenated benzoquinones tested in this study oxidized  $Q_{400}$  just in the complete darkness. Furthermore, mid point potential of  $Q/Q^-$  does not account for the difference in the potency to oxidize  $Q_{400}$  between 2,5- and 2,6-DCBQs, since their mid point potentials are different only by 2 mV [16]. The redox potential of fluoranil is also high enough to oxidize  $Q_{400}$  as those of other tetrahalogenated benzoquinones [11]. Thus, besides the redox potential, another factor should be considered for the explanation of the present results. A steric hindrance and/or differences in binding positions of benzoquinone derivatives at the  $Q_B$  site might be the cause of the differences in their abilities to oxidize  $Q_{400}$ . However, we do not have any experimental data to support this speculation. Further experiments are needed to elucidate the mechanism of  $Q_{400}$  oxidation by halogenated benzoquinones in the dark.

It has also been reported that some halogenated benzoquinones increased the pool size of electron acceptors two- to four-fold [17]. This was attributed to covalent binding of the quinones on the reducing side of PS II [18, 19]. It was supposed that quinones did not share the binding site with DCMU but the quinones were not accessible to their site when DCMU occupied its own binding

site. Taking the present findings into consideration, these phenomena can be reinterpreted as follows: 1) Halogenated benzoquinones oxidize  $Q_{400}$  in the dark. 2)  $Q_{400}$  oxidation results in a decrease in the binding affinity of DCMU to the  $Q_B$  site as reported by Wraight [3]. 3) By illumination,  $Q_{400}^+$  is rereduced which in turn increases the affinity of DCMU to the  $Q_B$  site causing complete inhibition of electron transport through  $Q_B$ . 4) Incubation with DCMU prior to addition of benzoquinones inhibits the oxidation of  $Q_{400}$  by the quinones so

that the work integral is not increases. Thus, an increase in the pool size more than two and the inaccessibility of benzoquinones to the binding site when the quinones are added after DCMU can be explained more reasonably. However, it must be also true that some amounts of benzoquinones bind covalently to PS II [19]. It is necessary to re-investigate the effects of halogenated benzoquinones especially those of tribromotoluquinone in more detail.

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