Interaction of Halogenated 1,4-Benzoquinones with System II of Photosynthesis

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The interaction of halogenated *p*-benzoquinones with PS II has been analyzed by measurements of fluorescence induction curves and the average oxygen yield per flash in isolated class II chloroplasts. It was found:

1) The normalized area over the fluorescence induction curve in the presence of DCMU, A/F_{max}, markedly increases if halogenated *p*-benzoquinones are added before DCMU. The effect is eliminated by DCMU addition prior to that of the quinones.
2) The extent of A/F_{max} increases with increasing dark time between the additions of 2,3,5-tribromo-6-methyl-1,4-benzoquinone (TBTQ) and DCMU, respectively.
3) Some of the halogenated *p*-benzoquinones were found to act as efficient electron acceptors under repetitive excitation at low flash frequency (2 Hz). In the case of TBTQ and 2,3-dichloro-5-t-butyl-1,4-benzoquinone (TBU 13) the sensitivity of the average oxygen yield per flash was shifted towards higher concentrations of DCMU.
4) Halogenated *p*-benzoquinones can also affect the stability of oxidizing redox equivalents in the water oxidizing enzyme system Y. This effect depends on the nature of the substituents.

The present results are interpreted by the assumption of a covalent binding of halogenated *p*-benzoquinones in the vicinity of Q_A. This binding is prevented by DCMU. The possibility of allosteric interaction between the donor and acceptor side of PS II is discussed.

Introduction

Many herbicides interrupt the electron transport between the primary (Q_A) and secondary (Q_B) plastoquinone at the PS II acceptor side (for review see ref. [1, 2]). Based on proteolytic degradation experiments a proteinaceous component was inferred to function as apoprotein of these functional quinones which simultaneously acts as target for noncovalent herbicide binding and as shield to exogenous redox substances [3, 4]. PS II herbicides were assumed to function predominantly via an allosteric mechanism. An alternative model proposed a direct competition of herbicides with the binding of Q_B [5, 6]. This model implies that a plastoquinone molecule becomes tightly bound to the Q_B binding site only in its semiquinone form, but is rather easily exchangeable in the quinone and quinol redox state. Binding and replacement studies with radioactively labeled herbicides as well as electron accepting quinones supported the competitive model to some extent [7, 8] but other data indicated that allosteric effects are also of functional relevance [9, 10]. The interaction of quinone derivatives is of special interest because these substances resemble native quinones in their ability to act as redox active components but in addition some of them can also inhibit the electron transport at PS II [11]. In this communication the interaction of halogenated 1,4-benzoquinones with PS II has been analyzed to attack two problems:

1) Do these compounds bind in the vicinity of Q_A, so that a DCMU-insensitive electron transport can take place from Q_A^- to the bound quinones?

Abbreviations: *A*, area over the induction curve; p-BQ, *p*-benzoquinone; Chloranil, 2, 3, 5, 6 tetrachloro-1,4-benzoquinone; D_1, 32 kDa Q_B-protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_{max}, maximal fluorescence intensity; MES, 2-(N-morpholino) sulfonate; TBTQ, 2,3,5-tribromo-6-methyl-1,4-benzoquinone; TBU 13, 2,3-dichloro-5-t-butyl-1,4-benzoquinone; Tricine, tris (hydroxymethyl) methylglycine.

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2) Do these quinones compete for the binding site of DCMU?

These questions were analyzed by means of fluorometric and amperometric methods.

Materials and Methods

Thylakoids (class II chloroplasts) were isolated from spinach by the method described in ref. [12], except that 10 mM ascorbate was present in the grinding medium. 5% dimethylsulfoxide was added for storage in liquid nitrogen. Fluorescence induction curves were measured at room temperature with an equipment similar to that described previously [13], except of using a sample holder for the cuvette instead of that for leaves. The reoxidation of the primary quinone acceptor, Q_A, in the presence of DCMU and NH_2OH was measured via changes of the fluorescence yield as outlined in ref. [14].

The chloroplast suspension contained: 5 μg chlorophyll/ml, 2 mM NaCl, 2 mM $MgCl_2$ and 50 mM Tricine NaOH pH = 7.5. The samples were completely dark adapted before starting the measurements. Quinones were added as indicated in the figure legends. The average oxygen yield per flash was measured with a Clark type electrode as described in ref. [15]. In this case the chloroplast suspension contained 50 μg chlorophyll/ml, 10 mM KCl, 2 mM $MgCl_2$ and 20 mM MES-NaOH, pH = 6.5 of exogenous electron acceptor were added as indicated in the figure legends. The synthesis of the 1,4-benzoquinones is described in ref. [16].

Results

In order to test the possibility of the binding of halogenated 1,4-benzoquinones in the vicinity of Q_A fluorescence induction curves were measured in the presence of DCMU without and with the corresponding quinone. A typical trace of the curves obtained is depicted in Fig. 1. Generally two effects arise if the samples are incubated in the dark with halogenated 1,4-benzoquinones 5 min before DCMU addition: a) the maximum level of the fluorescence decreases due to the well established non-photochemical quenching effect of quinones [17]. This effect which is not specific for halogenated quinones also occurs if quinones are added after DCMU. b) The area over the fluorescence induction curve, A, normalized to the maximum level, F_{max},

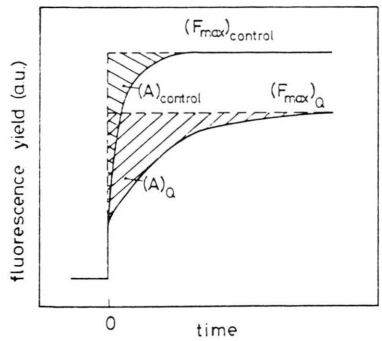
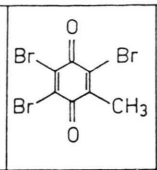
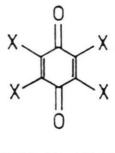
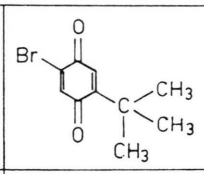
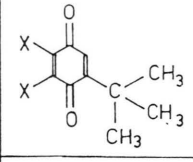


Fig. 1. Schematic representation of fluorescence induction curves in thylakoids in the absence and presence of halogenated 1,4-benzoquinones and DCMU. Q = quinone.

markedly increases due to incubation with halogenated 1,4-benzoquinones. This effect disappears if DCMU is added before the quinones (vide infra). The area over the induction curve normalized to the maximum fluorescence, A/F_{max}, represent the acceptor capacity for electrons [18]. As the capacity of the control in the presence of DCMU is one electron, the ratio $(A/F_{max})_Q/(A/F_{max})_{control}$ reflects the total DCMU intensitive number of electrons that can be stored at the PS II acceptor side. The data summarized in Table I indicate that in the presence of DCMU 2–4 electrons can be transferred to the halogenated 1,4-benzoquinones. This amount does not correlate with the redox potential and the steric parameters (see [11]) of the different quinone derivatives. The increase of the normalized area over the fluorescence induction curve is not observed for non-halogenated quinone derivatives (data not shown). The most simple explanation for this phenomenon can be offered by the assumption that 1–2 halogenated quinones are bound in the vicinity of Q_A^- so that a sufficiently rapid electron transfer can take place. The effect saturates with increasing quinone concentration as is shown in Fig. 2. A completely different pattern however is observed if the halogenated quinones are added after DCMU. In this case no increase of A/F_{max} arises (see Fig. 2). This result indicates that DCMU prevents quinone binding in the vicinity of Q_A. On the other hand quinone binding does not eliminate the blockage of electron transport by DCMU. In order to understand these effects it appears reasonable to assume that halogenated quinones bind irreversibly at a site which is different from that for DCMU. This idea is in line with latest

Table I. Effects of various halogenated 1,4-benzoquinones on the normalized area over the fluorescence induction curve $(A/F_{max})_Q/(A/F_{max})_{control}$.

compound				
$\frac{(A/F_{max})_Q}{(A/F_{max})_{control}}$	4.3 (3 μM)	X = Cl 2.5 (1 μM) X = Br 3.2 (1 μM) X = I 3.1 (1 μM)	4.3 (1 μM)	X = Cl 3.2 (3 μM) X = Br 4.5 (0.5 μM) X = I 3.7 (1 μM)

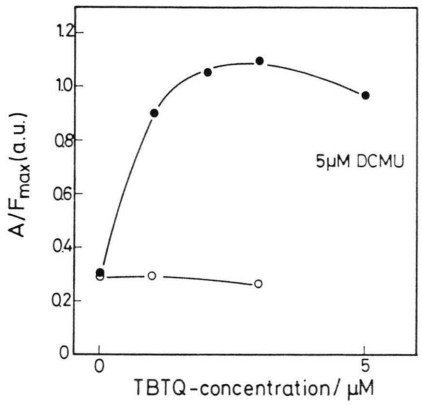


Fig. 2. Normalized area over the fluorescence induction curve, A/F_{max}, as a function of TBTQ-concentration in thylakoids. ●, 5 min dark incubation at the indicated TBTQ concentration prior to addition of DCMU to a final concentration of 5 μM; ○, DCMU addition before TBTQ. Other experimental details as in Materials and Methods.

findings of a covalent binding of [^{14}C]tetrabromo-1,4-benzoquinone to a 41 kDa polypeptide in PS II [19]. On the basis of the above mentioned interpretation the replacement of halogenated 1,4-benzoquinones is expected to depend on the time elapsed between additions of the quinone and of DCMU, respectively. In Fig. 3 the normalized area over the fluorescence induction curve $(A/F_{max})_Q/(A/F_{max})_{control}$ is depicted as a function of the dark incubation time with 2,3,5-tribromo-6-methyl-1,4-benzoquinone (TBTQ) before addition of DCMU. The data show an increase of the normalized area over the fluorescence induction curve with increasing dark incubation reaching the maximum value after 2–3 min. This time course can be interpreted as the binding kinetics of TBTQ.

The data reported so far suggest that TBTQ binds covalently in the vicinity of Q_A and that this effect is prevented in the presence of DCMU. This implies that a mutual interaction does exist between the binding sites of DCMU and TBTQ, respectively. Accordingly, after covalent TBTQ-binding also the affinity of DCMU is expected to be affected. In order to test this idea the effect of DCMU on the electron transport was analyzed in control and TBTQ treated samples by measuring the average oxygen yield per

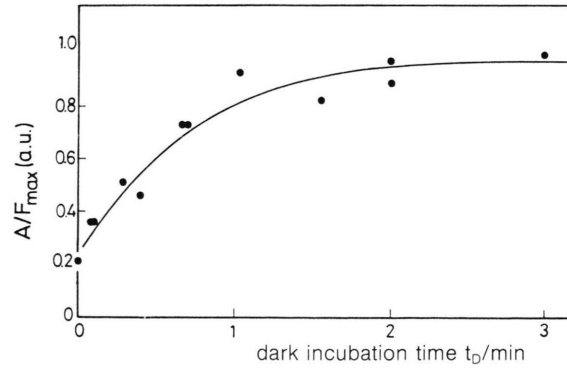


Fig. 3. Normalized area over the fluorescence induction curve, A/F_{max}, as a function of the dark incubation time t_D between addition of TBTQ (3 μM) and DCMU (5 μM), respectively, in thylakoids. Other experimental details as in Materials and Methods.

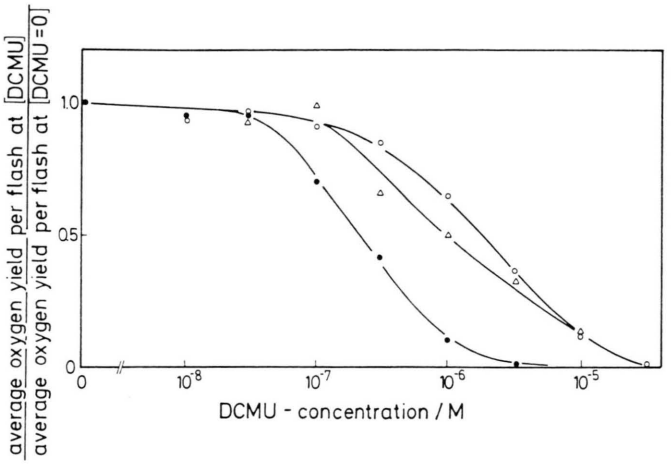


Fig. 4. Average oxygen yield per flash as a function of DCMU-concentration in thylakoids. Electron acceptors: ●, 100 μM *p*-benzoquinone; △, 100 μM 2,3-dichloro-t-butyl-1,4-benzoquinone; ○, 100 μM TBTQ. Time between the flashes 250 ms. Other experimental conditions as described in Material and Methods.

flash as a function of DCMU concentration. The results obtained are depicted in Fig. 4. It is shown that TBTQ decreases the susceptibility of oxygen evolution to DCMU by about one order of magnitude. This phenomenon is only observed if TBTQ is added before DCMU (data not shown).

Besides of the specific binding effect of halogenated 1,4-benzoquinones the possibility should be taken into account that these compounds could also interact in the presence of DCMU with Q_A^- without covalent binding in its vicinity. To check this possibility experiments were performed in thylakoids that were pretreated with DCMU and NH_2OH. In this case the back reaction between Q_A^- and S_2 of the water oxidizing enzyme system Y is prevented so that Q_A^- exhibits a very long life time [20]. The reoxidation of Q_A^- was measured via the relaxation of the flash induced fluorescence yield accounting for the nonlinear relation between Q_A^- (t) and F(t) by a procedure outlined previously [14, 21]. It is known that after one saturating flash the maximum fluorescence yield is not reached. Therefore the samples were illuminated with 25 flashes and the fluorescence decay was measured after the last flash. The data obtained are shown in Fig. 5. In the absence of added quinones Q_A^- exhibits the expected high stability. After addition of TBTQ the decay kinetics become accelerated. The relaxation rate increases with increasing TBTQ concentration. It is interesting to note that this effect does not disappear if DCMU is added before TBTQ (data not shown). This result indicates that TBTQ can interact with Q_A^- also under conditions where binding in its vicinity is prevented by DCMU. However, it has to be emphasized that in this case the electron transfer from Q_A^- to TBTQ is slower by orders of magnitudes than under conditions where TBTQ is bound in the neighbourhood of Q_A^-. The data of Fig. 5 exhibit another effect. Based on the multiphasic decay kinetics a heterogenous type of interaction between Q_A^- and TBTQ appears to be likely. However, this phenomenon will not be further analyzed in this communication. A last point should be considered for the interaction between TBTQ and the PS II acceptor side. As the polypeptides of the PS II core complex span the thylakoid

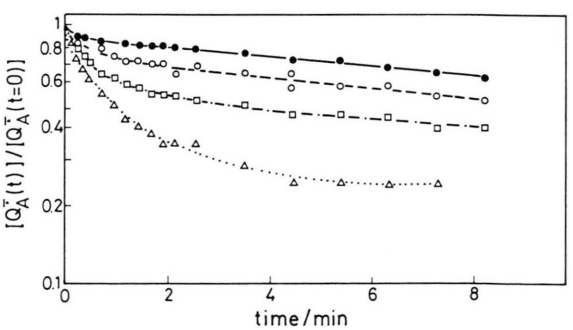


Fig. 5. Relative extent of reduced Q_A^- as function of time after excitation with 25 flashes of thylakoid in the presence of 2 μM DCMU and 500 μM NH_2OH at different TBTQ concentrations. ●, Control; ○, 3 μM; □, 6 μM; △, 12 μM. The suspension contained 20 μg chlorophyll/ml. Other experimental conditions as described in Material and Methods.

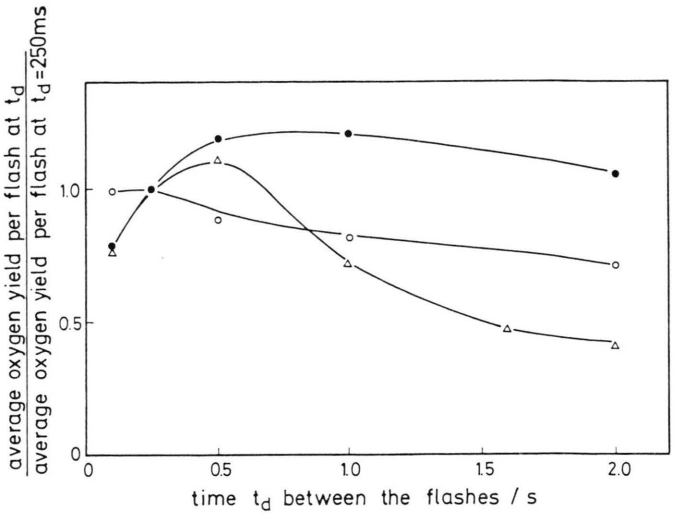


Fig. 6. Average oxygen yields per flash as a function of time between the flashes in thylakoids in the presence of different quinones. ○, 100 μM TBTQ; ●, 100 μM 2,3-dibromo-5-t-butyl-1,4-benzoquinone; △, 100 μM chloranil. Other experimental conditions as described in Material and Methods.

membrane structural modifications at the acceptor side could also indirectly change the reaction pattern at the donor side. In this respect the stability of oxidizing redox equivalents in the water oxidizing enzyme system Y is a parameter that could be easily affected. Informations about the stability of the intermediary redox states are obtainable by measuring the average oxygen yield per flash as a function of the time between the flashes [15]. Different halogenated 1,4-benzoquinones were tested. The data obtained (see Fig. 6) reveal that effects on the water oxidizing enzyme system are strongly dependent on the type of the substituents. A similar effect has been reported previously for substituted diphenyl-amines [23].

Discussion

The results presented in this study provide evidence for a covalent binding of halogenated 1,4-benzoquinones in the vicinity of Q_A which permits rapid Q_A^--reoxidation after preillumination in the presence of DCMU. This quinone binding is prevented by DCMU.

The first indication for the interaction of an exogenous quinone with PS II has been reported previously [24] for unsubstituted *p*-benzoquinone (p-BQ). In this case, however, the *p*-benzoquinone becomes sufficiently tightly bound only if its reduced semiquinone form is generated by electron transfer from Q_B^- to p-BQ. No binding was observed in the dark. In contrast to p-BQ in this study halogenated *p*-benzoquinones were found to bind in the dark. This suggests a different type of binding mechanism. Based on latest findings [19] a covalent binding seems to take place of the halogenated *p*-benzoquinones reacting as vinylogous acid halides with nucleophilic residues of the protein in a Michael-type addition/elimination mechanism.

The binding of quinones raises the question about the target polypeptide. As the 32 kDa atrazine binding protein D1 is inferred to contain the Q_B-binding site [25], one might expect that halogenated *p*-benzoquinones compete with the endogenous plastoquinone for the native site of Q_B. This however does not seem to be the case because latest experiments with a ^{14}C-labeled tetrabromo-1,4-benzoquinone revealed that a 41 kDa polypeptide of PS II becomes predominantly tagged but not the 32 kDa Q_B-protein. If one assumes that the results reported in Fig. 1–3 of this study are due to quinone binding to the 41 kDa polypeptide then that part containing the binding site should be in close contact with Q_A because of the rapid electron transfer from Q_A to these quinones. Furthermore, it has to be taken into account that DCMU prevents the binding of halogenated *p*-benzoquinones. The inhibitory effect could be explained by a modification of the Q_B-site due to binding of the exogenous quinones. This probably leads to a marked retardation of the electron transport rate under continuous illumination rather than to a complete blockage of Q_A-reoxidation. This idea

is supported by the finding that under repetitive excitation at low flash frequency (2 Hz) the average oxygen yield declines only at rather high concentrations. Some of the halogenated *p*-benzoquinones (*e.g.* TBTQ) even act as efficient electron acceptors under these conditions. Another effect which should be briefly mentioned is the slight destabilization of oxidizing redox equivalents in the water oxidizing enzyme system Y by a few derivatives (*e.g.* chloranil). This phenomenon reflects allosteric interaction between donor and acceptor side reactions. In this respect it is interesting to note that partial inhibition of electron transport by DCMU leads to reduction of the life time of the intermediary redox states in system Y [26, 27]. As the effect disappears after trypsin treatment of the thylakoids [23] an allosteric mechanism appears to be reasonable. This result would be in line with the assumption that D-1 does not only contain the binding site for DCMU but also participates in the ligation of the functional manganese in system Y [28]. Likewise other classes of substances like diphenylamines also affect the donor side [23]. However in the latter case, the effect on system Y does not disappear after trypsin treatment [23]. These results indicate that there do exist mechanistic differences. The implication of these data for the structural and functional organization of PS II and the interaction of herbicides (for recent discussions see ref. [29–31]) have to be analyzed by further more detailed mechanistic studies.

Acknowledgements

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