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Interaction of 1,4-benzoquinones with Photosystem II in thylakoids and Photosystem II membrane fragments from spinach

G. Renger [a], B. Hanssum [a], H. Gleiter [a], H. Koike [b] and Y. Inoue [b]

[a] *Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität, Berlin (Germany) and* [b] *Solar Energy Research Group, The Institute of Physical and Chemical Research, Riken, Wako-shi, Saitama (Japan)*

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The interaction of exogenous quinones with the Photosystem II (PS II) acceptor side has been analyzed by measurements of flash-induced 320 nm absorption changes, transient flash-induced variable fluorescence changes, thermoluminescence emission and oxygen yield in dark-adapted thylakoids and PS II membrane fragments. Two classes of 1,4-benzoquinones were shown to give rise to remarkably different reaction patterns. (A) Phenyl-*p*-benzoquinone (Ph-*p*-BQ) -type compounds give rise to a marked binary oscillation of the initial amplitudes of 320 nm absorption changes induced by a flash train in dark-adapted PS II membrane fragments and a retardation of the decay kinetics of the flash-induced variable fluorescence. The electron transfer reactions to these type of quinones are severely inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). (B) In the presence of tribromotoluquinone (TBTQ) a different oscillation pattern of the 320 nm absorption changes is observed characterized by a marked relaxation after the first flash in the 5 ms domain. This relaxation is insensitive to 10 μM DCMU. Likewise the decay of the flash-induced variable fluorescence in TBTQ-treated samples is much less sensitive to DCMU than in control. The thermoluminescence emission exhibits an oscillation in samples incubated for 5 min with TBTQ before addition of 30 μM DCMU. Under the same conditions a significant flash-induced oxygen evolution is observed only after the third and fourth flash, respectively, whereas in the presence of TBTQ alone a normal oscillation pattern is observed. The different functional patterns of PS II caused by the two types of classes of exogenous quinones are interpreted by their binding properties: a noncovalent association with the Q_B-site of Ph-*p*-BQ-type quinones versus a tight (covalent?) binding in the vicinity of Q_A (possibly also at the Q_B-site) in the case of halogenated 1,4-benzoquinones. The mechanistic implications of these findings are discussed.

Abbreviations: D-1, 32 kDa herbicide binding protein of Photosystem II reaction center; D-2, polypeptide (30–34 kDa) of Photosystem II reaction center; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DIDS, diisothiocyanostilbene-2,2-disulfonic acid; F_i, initial fluorescence; LCC, lauroylcholine chloride; P-680, photoactive chlorophyll *a* of Photosystem II reaction center; Ph-*p*-BQ, phenyl-*p*-benzoquinone; PS-II, Photosystem II; Q_A and Q_B, primary and secondary plastoquinone acceptor of Photosystem II; S_i, redox states of the catalytic site of water oxidation; TBTQ, tribromotoluquinone. Mes, 4-morpholineethanesulfonic acid.

Correspondence: G. Renger, Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität, Sekr. PC 14, Strasse des 17. Juni 135, D-1000 Berlin 12, Germany.

Introduction

Photosynthetic water cleavage takes place in a membrane-bound complex containing at least seven polypeptides [1]. The overall process comprises three reaction sequences (for a recent review, see Ref. 2): (a) generation of oxidizing and reducing redox equivalents initiated by the ejection of an electron from the lowest excited singlet state of a special chlorophyll *a*, referred to as P-680 and its transfer to a specifically bound plastoquinone Q_A; (b) water oxidation to dioxygen via a four-step univalent reaction sequence at a manganese-containing catalytic site; and (c) plastoquinone reduction to plastoquinol via a two-step univalent reaction sequence at a special binding niche (designated as Q_B-site) in polypeptide D-1.

The mechanism of the latter process is of interest for many reasons because the binding niche does not only interact with its substrate but it is also susceptible to many substances that interrupt the electron transport by competitive and/or allosteric blockage of PQ-reduction and therefore these compounds are of practical relevance as potent herbicides (for a recent review, see Ref. 3). Among the great variety of chemicals affecting the Q_B-site exogenous quinones are especially interesting as analogues of the native substrate because their structural and redox properties can be varied over a wide range. Accordingly, these compounds should provide a sensitive tool to monitor the mode of interaction with the Q_B-site (and/or alternative binding sites) and its effect on the electron transfer from Q_A^- to these quinones. In the native state plastoquinone moderately bound to the Q_B-site accepts an electron from Q_A^- thereby attaining the redox level of a semiquinone which is tightly bound and highly stabilized [4] in the Q_B-site. After a second electron transfer from Q_A^- and protonation plastoquinol is formed. This PQH_2 has low binding affinity and becomes released from the Q_B-site by exchange with another PQ. A high spin Fe^{2+} coordinatively bound to the reaction center complex is located between Q_A and Q_B. The functional role of this Fe^{2+} remains to be clarified.

Recently some exogenous quinones were shown to induce the oxidation of Fe^{2+} to Fe^{3+} after a single turnover flash. This phenomenon was interpreted as a reductant induced oxidation of Fe^{2+} by the semiquinone form of these compounds which is generated at the Q_B-site through electron transfer from Q_A^- [5,6]. DCMU eliminates this effect.

Another interesting phenomenon was observed for halogenated 1,4-benzoquinones. These quinones were inferred to bind tightly (probably covalently) in the vicinity of Q_A [7,8]. The bound quinones(s) is (are) able to accept electrons from Q_A^- in a DCMU-intensitive transfer reaction, whereas preincubation with DCMU prevents the binding at a site which permits reoxidation of Q_A^- [7]. These findings show that different classes of quinones exhibit a quite different interaction pattern with PS II. Using phenyl-1,4-benzoquinone and tribromotoluquinone as prototypes the present study analyzes the different modes of interaction of exogenous quinones with the PS II acceptor side in more detail.

Materials and Methods

Thylakoids were prepared from spinach according to the method of Winget et al. [9], except that 10 mM ascorbate was present in the grinding medium. For the isolation of PS II membrane fragments the procedure of Berthold et al. [10] was used with some modifications described in Ref. 11.

The standard reaction mixture for measurements of UV-absorption changes contained: PS II membrane fragments (10 μg chlorophyll per ml), 10 mM NaCl, 50 mM Mes/NaOH (pH = 6.0; or pH = 6.5) or 50 mM Tricine/NaOH (pH = 7.5) and electron acceptor as indicated in figure legends.

The standard reaction mixture for fluorescence and thermoluminescence measurements contained: thylakoids (500 μg chlorophyll/ml), 20 mM NaCl and 20 mM MES/NaOH (pH = 6.0); other additions as indicated in the figure legends. For the fluorescence measurements the reaction mixture was incubated in the dark for at least 5 min before 20-fold dilution in order to obtain a final chlorophyll concentration of 10 μg/ml.

Flash-induced UV-absorption changes were detected in dark-adapted samples with an

equipment described previously [12]. Thermoluminescence emission was monitored with a set-up described in Ref. 13.

The measurements of flash-induced changes of fluorescence yield were performed with a newly constructed fluorometer [14] which resembles in its basic principles that described in Ref. 15. In order to achieve a high time resolution, the samples were excited with saturating pulses from a Nd/YAG laser (λ = 532.8 nm, E = 3–5 mJ/pulse). The fluorescence changes were detected via a modulation technique using high-frequency trains of weak LED-pulses (duration, 1 μs) as nondisturbing monitoring system. The fluorescence emission caused by these very weak LED-pulses was detected by an avalanche photodiode (RCA C 30872). The time resolution of the equipment is of the order of 1 μs. The data were transiently stored in a Nicolet 1147 and further numerical processing was performed.

The time between the flashes was 1 s in all measurements, except of the oxygen yield measurements (t_d = 300 ms).

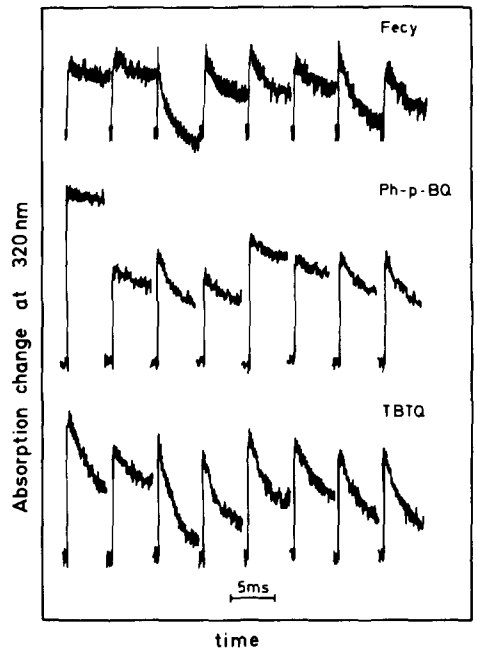


Fig. 1. Absorption changes induced at 320 nm by a train of laser flashes in dark-adapated PS II membrane fragments at pH = 6.0. Traces a were obtained in the presence of 1 mM $K_3[Fe(CN)_6]$; b in the presence of 100 μM Ph-*p*-BQ and c in the presence of 10 μM TBTQ. Other experimental conditions were as described in Materials and Methods.

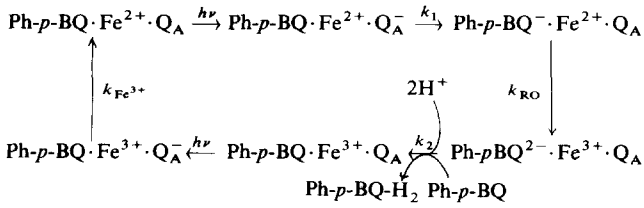
Results

Oscillation pattern of flash-induced absorption changes at 320 nm as a function of different acceptors in PS II membrane fragments

The interaction of different exogenous redox couples with PS II was analyzed by measuring absorption changes at 320 nm induced by a flash train in dark-adapted PS II membrane fragments from spinach. At 320 nm the difference spectra of Q_A^-/Q_A and Q_B^-/Q_B exhibit pronounced maxima [16,17]. Therefore information can be obtained about the reaction pattern of the PS II acceptor side provided that contributions are properly separated which arise due to the turnover of the catalytic site of water oxidation [18,19]. Fig. 1 shows flash-induced 320 nm absorption changes in dark-adapted samples in the presence of $K_3[Fe(CN)_6]$, phenyl-*p*-benzoquinone (Ph-*p*-BQ) and tribromotoluquinone (TBTQ). It is obvious that quite different oscillation patterns are observed. As in all cases the water-oxidizing enzyme system remains almost unaffected by these chemicals (data not shown), and the differences predominantly reflect a different mode of interaction with the PS II acceptor side. Two striking phenomena are observed: (a) marked dependence on the acceptor type in the amplitude and kinetics of the absorption change caused by the 1st flash, ΔA_1^{320} (0); and (b) the binary oscillation of the initial amplitude in the presence of Ph-*p*-BQ which is less pronounced in the case of TBTQ and is absent when $K_3[Fe(CN)_6]$ is present.

The decrease of the initial amplitude of ΔA_1^{320} by $K_3[Fe(CN)_6]$ is due to the dark oxidation of the iron located between Q_A and Q_B [6], because Fe^{3+} provides an endogenous acceptor for rapid electron transfer from Q_A^- to Fe^{3+} which is not resolved in the 5 ms time domain. The decrease of ΔA_1^{320} (0) is not observed if 10 μs flashes are used for excitation (data not shown) because in this case all centers containing oxidized Fe^{3+} undergo a double turnover [20].

In terms of the mode of interaction of exogenous quinones with the PS II acceptor side the striking differences between Ph-*p*-BQ and TBTQ are of special interest. In the case of Ph-*p*-BQ the



(1)

marked binary oscillation of the initial amplitudes ΔA_n^{320} (0) with flash number n has been interpreted [21] by the model of a reductant induced oxidation of Fe^{2+} with Ph-p-BQ^- bound to the Q_B-site as oxidant [5,6]. This reaction can be described by Eqn. 1, where the rate constants k_1 and $k_{Fe^{3+}}$ refer to Q_A^- reoxidation by Ph-p-BQ and Fe^{3+}, respectively, and k_{RO} describes the reductant induced oxidation of Fe^{2+} by Ph-p-BQ^-. The rate constant k_1 (in vivo—with PQ instead of Ph-p-BQ—half-life times of 100–400 μs were detected, see Refs. 22 and 23) is decreased in the presence of Ph-p-BQ compared with PQ as shown by fluorescence measurements (Fig. 2). If one takes into account the large absorption differences between states Ph-p-BQ^{2-} $Fe^{3+}Q_A$ and either Ph-p-BQ Fe^{2+} Q_A^- or Ph-p-BQ^- $Fe^{2+}Q_A$ [24] the data of Fig. 1 clearly indicate that the Fe^{2+} oxidation according to Eqn. 1 requires half times of at least 20 ms, because no relaxation of the absorption change induced by the first flash ΔA_1^{320} is observed in the 5 ms time domain. A strikingly different pattern is observed in the presence of TBTQ, where ΔA_1^{320} (t) exhibits a marked relaxation within the 5 ms time domain (vide infra) and the binary oscillations of A_n^{320} (0) are less pronounced. This behaviour suggests a different reaction mechanism of TBTQ compared with Ph-p-BQ. One essential difference between both quinone types is the possibility of tight (probably covalent) binding of TBTQ close to Q_A inferred from binding studies [7] and measurement of fluorescence induction curves [8]. Therefore, the effect of TBTQ was analyzed more thoroughly especially in comparison with Ph-p-BQ as a reference. A parameter very sensitive to modifications of the functional coupling of Q_A^- with bound quinones (at Q_B-site or another place close to Q_A) is the rate of Q_A^- reoxidation. The reoxidation of Q_A^- can be monitored by measuring the relaxation kinetics of the flash-induced fluorescence yield enhancement (variable fluorescence) [22].

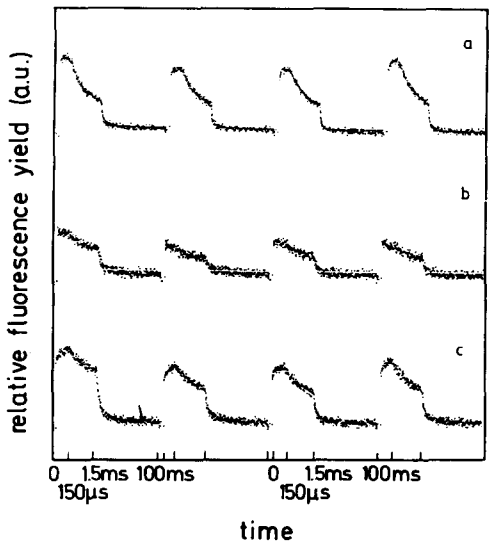


Fig. 2. Transient changes of fluorescence yield induced by a train of four laser flashes in dark adapted thylakoids at pH = 6.0. Traces a: Control without acceptor; traces b: in the presence of 25 μM Ph-p-BQ and traces c: 30 μM TBTQ. The measurements were performed at a chlorophyll concentration of 10 μg/ml. Other experimental conditions were as described in Materials and Methods.

Kinetics of flash-induced change of fluorescence yield at different exogenous acceptors in dark-adapted thylakoids

The properties of the PS II acceptor side were found to be affected by the Triton X-100 solubilization used for the isolation of PS II membrane fragments [25]. Therefore, the fluorescence experiments were performed in normal thylakoids. The results obtained are compiled in Fig. 2. In order to cope with a time domain of 100 ms and simultaneously to illustrate the extent of fast kinetics on the μs-time scale the signals were recorded at three different time axes as indicated in the figure (first part included 150 μs; second part, 1.5 ms).

In dark-adapted control samples illuminated with a train of four laser flashes an unresolved fast increase of fluorescence is followed by slower rise kinetics which reflect the μs-components of P-680$^+$ reduction [26] because P-680$^+$ acts as fluorescence quencher [27]. The relaxation is dominated by kinetics in the range of 300–600 μs. After 100 ms the flash-induced variable fluorescence completely relaxed. Interestingly, the variable fluorescence slightly oscillates in its extent during the flash sequence. This phenomenon reflects the differences in the quenching efficiency caused by the different redox states of the catalytic site of water oxidation [28,29]. Very interesting results are obtained in the presence of Ph-*p*-BQ and TBTQ. As quinones do not only function as electron acceptors but simultaneously also act as nonphotochemical fluorescence quenchers [30] the extent of the flash-induced variable fluorescence becomes markedly diminished thereby giving rise to a smaller signal-to-noise ratio. This effect is especially pronounced in the presence of Ph-*p*-BQ. Therefore, the experiments could be performed only at comparatively low Ph-*p*-BQ concentration. The poor signal-to-noise ratio of the data of Fig. 2b does not permit unambiguous conclusions about the extent of the initial amplitudes, but they clearly show a retardation of the fluorescence decay kinetics. This result indicates that Ph-*p*-BQ efficiently competes with the endogenous plastoquinone for the Q_B-site. Compared with the control, slower relaxation kinetics are also observed in the presence of TBTQ. The retardation of Q_A^- reoxidation gives rise to a reduced double-hit probability under excitation with xenon flashes of 15 μs duration as reflected in the oscillation pattern of oxygen evolution by the disappearance of the yield due to the second flash of the sequence (Messinger, J., Hanssum, B. and Renger, G., unpublished results). The oscillation of the maxima of the flash-induced fluorescence yield closely resembling the control pattern (compare Fig. 2a with Fig. 2c) are assumed to be due to donor side effects (vide supra).

A variation of the electron-transfer kinetics between redox groups bound to a protein matrix can originate either from structural modifications (change of distance and/or mutual orientation) or from a change of the redox gap [31,32]. In order to analyze more thoroughly the mode of interaction with the acceptor side the effect of DCMU was investigated on the oscillation pattern of 320 nm absorption changes and on the transient kinetics of the flash induced fluorescence yield in the

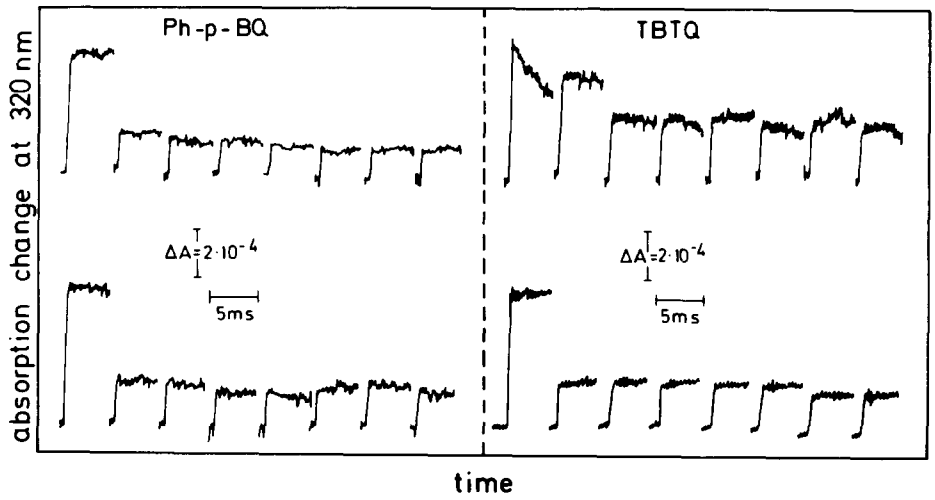


Fig. 3. Absorption changes induced at 320 nm by a train of xenon-flashes in dark adapted PS II membrane fragments at pH = 6.0. The bottom traces were obtained in samples incubated 5 min with 10 μM DCMU before addition of either 50 μM Ph-*p*-BQ or 10 μM TBTQ, the top traces were found if the quinones are added 5 min prior to DCMU. Other experimental conditions were as described in Materials and Methods.

presence of Ph-*p*-BQ and TBTQ respectively. The results are summarized in Figs. 3 and 4.

Effects of DCMU on the flash-induced pattern of 320 nm absorption changes in dark-adapted PS II membranes in the presence of different exogenous quinones

The top traces of Fig. 3 are obtained if samples are incubated with either Ph-*p*-BQ or TBTQ for 5 min in the dark before addition of DCMU. In the presence of Ph-*p*-BQ ΔA_1^{320} exhibits the normal extent whereas the absorption changes in the subsequent flashes are markedly reduced. In all cases the relaxation kinetics are slow. This pattern can be simply explained. The first flash gives rise to $S_2Q_A^-$ formation in all active Photosystems II. As Q_A^--reoxidation in the presence of DCMU can only occur via back reaction with S_2 the amplitudes in the subsequent flashes reflect the percentage of the systems that performed the reaction $S_2Q_A^- \rightarrow S_1Q_A$ during the dark time between the flashes, i.e., each absorption change ΔA_n^{320} reflects for $n \geq 1$ the same process: $S_1Q_A \xrightarrow{h\nu} S_2Q_A^-$. A significantly different pattern is observed if TBTQ is used instead of Ph-*p*-BQ: (i) the initial amplitude of the absorption change due to the first flash, ΔA_1^{320} (0) and its marked relaxation during a 5 ms time period remain practically unaffected, in contrast to the situation in the presence of Ph-*p*-BQ; (ii) the extent of the absorption change caused by the second flash of the sequence, ΔA_2^{320} (0), is strikingly larger than of the subsequent flashes; (iii) the amplitudes of the absorption changes ΔA_n^{320} (0), induced by the subsequent flashes ($n \geq 3$) are markedly smaller than ΔA_2^{320} (0). The ΔA_n^{320} (0) values exhibit a slight but discernible oscillation as a function of n; and (iv) at shorter dark time between the flashes (300 ms instead of 1 s) the absorption changes ΔA_1^{320} (t) and ΔA_2^{320} (t) remain virtually unaffected while the ΔA_n^{320} (0) values for $n \geq 3$ become reduced (data not shown).

The relaxation of the 320 nm absorption change after the first flash indicates a comparatively rapid electron transfer from Q_A^- (at least in a fraction of PS II) which is insensitive to DCMU (compare Fig. 1 with Fig. 3). This idea is supported by the finding of a rather large amplitude of ΔA_2^{320} (0) in the presence of DCMU. The initial amplitudes ΔA_n^{320} (0) for $n \geq 3$ are about 60–65% of ΔA_1^{320} (0) at $t_d = 1$ s between the flashes. This fraction is markedly higher than in samples containing Ph-*p*-BQ and DCMU where ΔA_n^{320} (0)/A_1^{320} (0) is only 30–35% for $n \geq 2$ (compare top traces of the left and right side of Fig. 3). At $t_d = 300$ ms, ΔA_3^{320} (0)/ΔA_1^{320} (0) declines to 30–35% in the case of TBTQ and to about 15% for samples with Ph-*p*-BQ. In a previous study the effect of TBTQ on the fluorescence induction curve was found to be absent if DCMU was added prior to TBTQ [8]. In order to check this point experiments were performed with samples preincubated in the dark with DCMU (5 min) before addition of either Ph-*p*-BQ or TBTQ. In the case of Ph-*p*-BQ the results are independent of the sequence of DCMU and quinone addition. This finding is in line with the idea of reversible noncovalent Ph-*p*-BQ binding to the Q_B-site and its competitive removal by DCMU. In contrast to that, the effect of TBTQ strongly depends on the order of addition of DCMU and TBTQ. It is largely prevented by preincubation with DCMU (compare traces at the right side of Fig. 3). If DCMU is added first, the pattern of the flash-induced absorption changes is practically the same for Ph-*p*-BQ and TBTQ whereas in the case of opposite sequence (TBTQ incubation before DCMU addition) a markedly different behaviour is observed. This phenomenon provides an independent line of evidence for strong TBTQ binding to the polypeptide at a site which is either occupied or significantly modified by DCMU [8].

In order to analyze the mutual interaction of DCMU and TBTQ in more detail comparative experiments were performed of the flash-induced variable fluorescence and its decay kinetics in intact thylakoids, of the thermoluminescence emission and of the oxygen evolution.

Effects of DCMU on flash induced changes of fluorescence yield of dark-adapted thylakoids in the presence of TBTQ

The data obtained are depicted in Fig. 4. In control samples (without TBTQ) in the presence of DCMU the fast rise due to Q_A^--formation and P-680$^+$-reduction is followed by a slow decay reflecting mainly the reaction $S_2Q_A^- \rightarrow S_1Q_A$ which cannot be accomplished during the dark time of 1

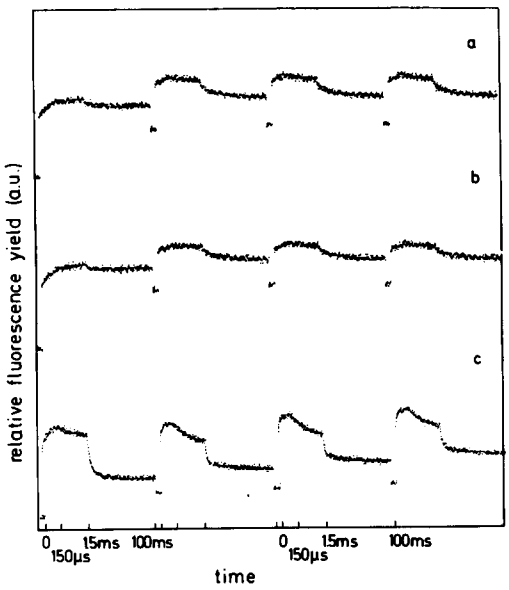


Fig. 4. Transient changes of fluorescence yield induced by a train of four laser flashes in dark adapted thylakoids at pH = 6.0. Traces a: control in the presence of 30 µM DCMU; traces b as a, but addition of 30 µM TBTQ 5 min after dark incubation with DCMU; traces c as a, but addition of 30 µM TBTQ in the dark 5 min prior to DCMU in the incubation medium (see Materials and Methods).

s between the flashes [33,34]. Therefore the flash induced fluorescence increase starts from a markedly higher F_i-level at the second and subsequent flashes. Similar traces of the transient flash-induced fluorescence yield were obtained if TBTQ is added after preincubation with DCMU (compare traces a and b). However, a strikingly different pattern is observed if TBTQ is added 5 min before DCMU. In this case the variable fluorescence induced by the first flash exhibits a substantial decay kinetics that are less pronounced in the subsequent flashes (Fig. 4c), but still of remarkably large extent compared with traces a and b. This result qualitatively corresponds with the measurements of 320 nm absorption changes (see Fig. 3). The nonlinearity between fluorescence quantum yield and the redox state of Q_A [35] has not been explicitly considered in this study because use of different sample types (thylakoids versus PS II membrane fragments) prevents a quantitative comparison with the 320 nm absorption changes (see Fig. 3). The traces of Fig. 4c indicate that after the first flash a comparatively fast DCMU-insensitive Q_A^- reoxidation takes place in more than 70% of PS II. After the subsequent flashes a slightly smaller extent of Q_A-recovery takes place in the 100 ms time domain.

Effects of TBTQ and DCMU addition on flash-induced thermoluminescence emission and oxygen yield

The data obtained from measurements of flash-induced 320 nm absorption changes and variable fluorescence in dark-adapted thylakoids (fluorescence) and PS II membrane fragments (ΔA^{320}) consistently show that TBTQ permits in both sample types a DCMU-insensitive Q_A^--reoxidation in the ms time domain after the first flash. The finding raises questions about the nature of the redox group accepting electrons from Q_A^-. To address this problem thermoluminescence measurements were performed. This method provides informations about the traps of oxidizing and reducing equivalents of PS II [36,37]. Typical traces of thermoluminescence after flash illumination of TBTQ-treated thylakoids and subsequent DCMU addition are depicted in Fig. 5. A comparatively low emission at the Q-band is observed after illumination with a single xenon flash, whereas after three flashes a markedly higher thermoluminescence arises at practically the same position. In the absence of DCMU no emission is observed (data not shown). Fig. 5 also reveals another interesting phenomenon. It shows that dark incubation of the sample on ice after additions of TBTQ and DCMU (5 min after TBTQ) causes an increase of the total emission. In contrast to that the opposite effect is observed in samples where DCMU addition preceded that of TBTQ. Fig. 6 presents a typical dependence of the thermoluminescence emission after one flash on the dark incubation of the treated sample on ice. A drastic reduction of the signal occurs. This phenomenon is due to TBTQ because in samples containing only DCMU the peak is hardly affected within a 30 min incubation at 0 °C (data not shown). The Q-band amplitude after short incubation (10 s) is very similar without or with TBTQ (not shown). The determination of thermoluminescence as a function of flash number in TBTQ/5 min/DCMU-treated samples is complicated by the enhancement of thermolumines-

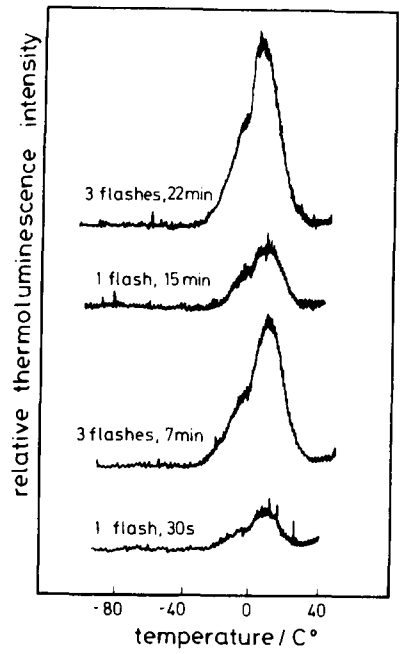


Fig. 5. Thermoluminescence bands induced by either one or three xenon flashes of spinach thylakoids incubated in the dark on ice at the indicated time after addition of 30 μM TBTQ and subsequent (5 min) addition of 30 μM DCMU. Flash excitation was performed at −15° C before rapid freezing of the samples. Experimental conditions were as described in Materials and Methods.

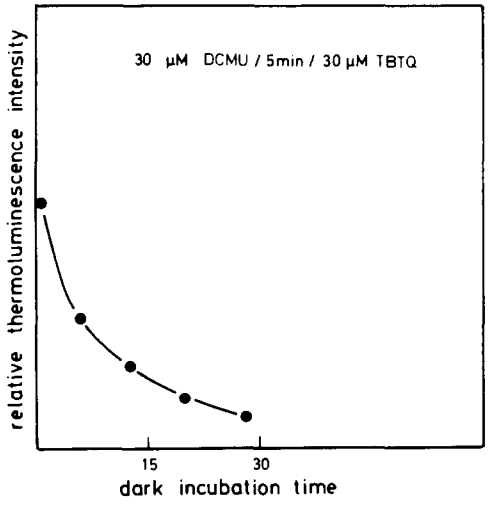


Fig. 6. Thermoluminescence intensity at peak emission as a function of dark incubation of thylakoids illuminated with one xenon flash at −15° C before rapid freezing. The samples were incubated in the dark with 30 μM DCMU prior (5 min) to addition of 30 μM TBTQ. The indicated incubation time starts after DCMU addition.

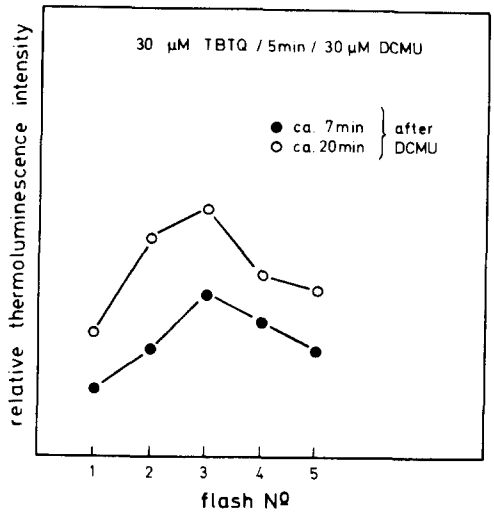


Fig. 7. Thermoluminescence intensity at peak emission as a function of the number of excitation flashes at −15° C in dark adapted thylakoids incubated for 5 min with 30 μM TBTQ prior to addition of 30 μM DCMU. 7 min (●) and 22 min (○) dark adaptation on ice after DCMU-addition. Other experimental conditions were as described in Materials and Methods.

cence emission with increasing dark incubation on ice. In order to eliminate this effect, five experiments were performed with each sample mixture over a period of approx. 30 min. The data obtained after illumination with one flash in the first, third and fifth experiment were used as reference values. Based on this method at two different incubation times similar oscillation patterns were observed as is shown in Fig. 7. Regardless of the unresolved mechanism of the above-mentioned enhancement at prolonged incubation (see Discussion) the results of Fig. 7 clearly show that the thermoluminescence emission after one flash is significantly smaller than after two or three flashes in TBTQ/5min/DCMU-treated thylakoids. The oscillation pattern of thermoluminescence emission suggests a possible participation of redox state transitions at the catalytic site of water oxidation in a fraction of PS II although at −15° C the reaction $S_3 \rightarrow (S_4) \rightarrow (S_0)$ is partially blocked [38]. In order to check for a possible redox turnover of the S_i-states in TBTQ/5 min/DCMU-treated samples the oscillation pattern of the oxygen yield induced by a flash train was measured with a Joliot-type electrode. The data obtained are depicted in Fig. 8. It shows that

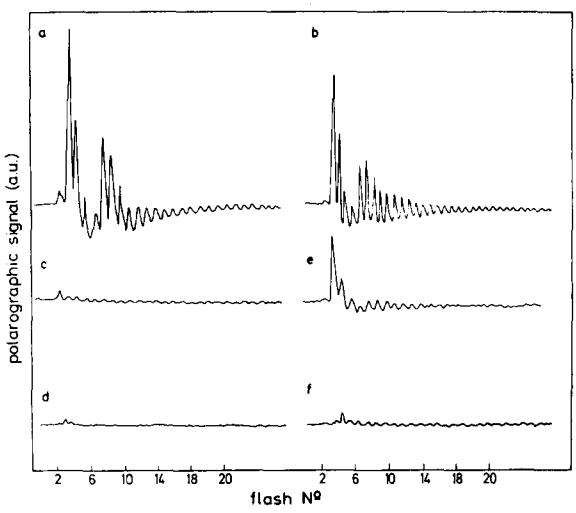


Fig. 8. Oxygen yield as a function of flash No. in dark adapted PS II membrane fragments. (a) Control; (b) in the presence of 50 μM TBTQ; (c) in the presence of 100 μM DCMU, (d) as (c) but addition of 50 μM TBTQ 5 min after DCMU. (e) 5 min dark incubation with 50 μM TBTQ before addition of 100 μM DCMU. (f) 100 μM Ph-*p*-BQ followed by 10 μM DCMU.

TBTQ alone does not significantly affect the oscillation pattern, except of an increase of the pool size (compare pattern a and b). Addition of DCMU to control samples completely suppressed oxygen evolution (trace c) and subsequent addition of 50 μM TBTQ is practically without effect (trace d). However, if TBTQ (50 μM) is added 5 min prior to 100 μM DCMU a significant oxygen yield is observed due to the third and fourth flashes while only marginal values are obtained in 7th and 8th flashes (compare traces a and e). Interestingly enough, the O_2-evolution in the TBTQ/5 min/DCMU samples almost disappears at a time of 1 s between the flashes (Messinger, J., Hanssum, B. and Renger, G., unpublished results). In contrast to TBTQ oxygen evolution is not supported by Ph-*p*-BQ in the presence of 50 μM DCMU (trace f).

Discussion

In this study the mode of interaction was analyzed between two types of 1,4-benzoquinones and the PS II-acceptor side, using Ph-*p*-BQ and TBTQ as prototypes of differently reacting species. Ph-*p*-BQ was confirmed to induce a mechanism of reductant induced oxidation of the endogenous Fe^{2+} [5,6] as reflected by the damped binary oscillation of the initial amplitudes of laser-flash-induced 320 nm absorption changes (the limited time resolution does not permit a detection of the electron transfer from Q_A^- to Fe^{3+}). Measurements of the flash-induced fluorescence yield exhibited significant differences of the maximum level only between the first and second flash because the highly damped oscillation in the subsequent flashes (see Fig. 1) is obscured due to the large quenching effect of Ph-*p*-BQ. The reactions of this quinone are highly sensitive to DCMU indicating a noncovalent association to the Q_B-site.

Similar phenomena – though less pronounced – were observed with some other quinones. Interestingly enough, the relaxation kinetics of the flash-induced fluorescence are slower in the presence of Ph-*p*-BQ compared with the control. This effect probably reflects suboptimal orientation and/or energy parameters (redox gap, reorganization energy) for electron transfer [31,32].

Markedly different reaction patterns are caused by TBTQ as the prototype of halogenated 1,4-benzoquinones [7,8]. A striking phenomenon is the partial relaxation in the 5 ms time domain of ΔA_1^{320} (t) after the first flash which is insensitive to DCMU. This effect is neither observed in the absence of an exogenous electron acceptor nor in the presence of $K_3[Fe(CN)_6]$ or nonhalogenated quinones [21]. The ms-relaxation very likely reflects a DCMU-insensitive Q_A^--reoxidation induced by TBTQ, probably in a bound form.

This effect obviously requires a specific binding of TBTQ. As the relaxation of flash-induced fluorescence is not drastically affected by TBTQ (compare Fig. 2a with Fig. 2c) and by subsequent addition of DCMU (compare Fig. 2c with Fig. 4c) TBTQ binding has to occur at a distance and orientation to Q_A that permits efficient reoxidation of Q_A^-. This raises the question about the nature of the TBTQ binding site. Taking into account that preincubation with DCMU largely prevents the TBTQ effect one might suggest that TBTQ becomes tightly bound directly at the Q_B-site, possibly by forming a covalent bond as discussed recently [8]. The latter idea is supported by recent findings of halogenated quinones being able to form covalent linkages to proteins [7,39]. However, different results reported in the literature

could be indicative for a more complex pattern. Binding studies with the chemically closely related ^{14}C-labeled compound bromanil revealed that a 41 kDa polypeptide rather than D-1 becomes predominantly tagged [7]. Furthermore, in contrast to atrazine, bromanil does not exhibit a Michaelis-Menten type saturation behaviour typical for a specific binding site. Although the preferential labeling of the 41 kDa polypeptide does not exclude the possibility that the minor extent of binding in the 30–34 kDa region (D-1) does really reflect that fraction of TBTQ which gives rise to the effect observed in this study an assignment of the 'functional' TBTQ to the Q_B site might be questionable for the following reasons: Bromanil does not affect the number of binding sites of atrazine, but only reduces their affinity significantly. A similar tendency was observed for TBTQ (Fromme, R. and Renger, G., unpublished results). Analogous results were obtained for either atrazine or ioxynil binding after covalent linkage of azido-quinone to thylakoids [40]. This behaviour favours an allosteric type of interaction between quinone and herbicide binding [41,42].

Regardless of the identification of the nature of its binding site different mechanistic aspects have to be considered for the reactions induced by TBTQ. As TBTQ highly reduces the affinity to binding of herbicides like atrazine and DCMU it appears reasonable to assume that in the presence of TBTQ the endogenous plastoquinone does not function as acceptor for Q_A^- reoxidation in a significant fraction of centers. This conclusion which is supported by the remarkable relaxation kinetics within the 5 ms domain of the 320 nm absorption change induced by the first flash implies a number of questions: (a) How is the linear electron transport sustained under repetitive flash excitation in the absence of DCMU? (b) What is the origin of the almost DCMU-insensitive relaxation kinetics after the first flash? (c) What is the mechanism of DCMU inhibition under repetitive flash excitation? The almost TBTQ invariant oscillation pattern of oxygen yield at a flash frequency of 2–4 Hz (compare Fig. 8, traces a and b) indicates that Q_A^- becomes efficiently oxidized after each flash. This effect could be either explained by a TBTQ exchange after its complete reduction or by covalently bound TBTQ molecule(s) that is (are) able to react with exogenous acceptor. The present data do not permit an unequivocal distinction between these alternatives. In respect to the second question different mechanisms can be considered for the interpretation of the relaxation kinetics of ΔA_1^{320} (t) within the 5 ms time domain. If bound TBTQ would resemble Ph-*p*-BQ by causing reductant induced oxidation of Fe^{2+} the relaxation could reflect a markedly faster oxidation step, i.e., k_{RO} of Eqn. 1 should be of the order of 150 s^{-1} in the presence of TBTQ. This idea, however, is hardly reconcilable with the flash-induced fluorescence measurements because extent and rise kinetics caused by the second flash are only slightly affected by TBTQ. If the iron center would be oxidized in a significant fraction of centers after the first flash the rise in the μs-range is expected to disappear and the maximum amplitude should be drastically reduced in the second flash because the fast fluorescence decay due to the electron transfer from Q_A^- to Fe^{3+} would overlap the rise kinetics. This is obviously not the case (compare Fig. 2, traces a and c). Therefore the relaxation of ΔA_1^{320} (t) in the 5 ms time domain more likely reflects Q_A^--reoxidation by TBTQ rather than a reaction $Q_A^- Fe^{2+}$ TBTQ + 2 $H^+ \rightarrow Q_A Fe^{3+}$ $TBTQH_2$. This conclusion implies again two alternative interpretations: either the difference extinction coefficient $\Delta\varepsilon(TBTQ^-/TBTQ)$ is significantly smaller than $\Delta\varepsilon(Q_A^-/Q_A)$ at 320 nm or TBTQ is bound in the form of a stable semiquinone in a fraction (20–30%) of dark-adapted centers. The latter idea would be in line with latest findings indicating that halogenated quinones like dichloro-1,4-benzoquinones form stable semiquinone radicals in the dark [43]. Unfortunately, no decision can be made for the former alternative because data about the difference spectrum of $TBTQ^-$/TBTQ for molecules bound to PS II are not available. In this respect it is interesting to note that the spectra of TBTQ and $TBTQH_2$ in solution markedly differ from those of PQ and PQH_2, respectively. Therefore, reliable conclusions cannot be drawn about the possible interpretations of ΔA_1^{320} (t) in the 5 ms domain reflecting either reaction

$$Q_A^- \; Fe^{2+} \; TBTQ^- \rightarrow Q_A \; Fe^{2+} \; TBTQ^-$$

or

$$Q_A^- \; Fe^{2+} \; TBTQ^- \xrightarrow{2\,H^+} Q_A \; Fe^{2+} \; TBTQ\,H_2$$

The data of this study show that bound TBTQ is able to accept electrons from Q_A^- in a DCMU-insensitive reaction. Accordingly, the inhibitory effect of DCMU after a few flashes is either due to rapid DCMU binding [44] after formation and release of TBTQ H_2 or it is caused by blockage of the reoxidation of bound reduced TBTQ by exogenous acceptors. The latter idea appears to be more likely because in the presence of DCMU even after more than 20 preflashes Q_A^--reoxidation is markedly faster in TBTQ-treated samples [8]. The possibility of covalent binding at the PS II acceptor side implies another interesting phenomenon for the interaction of TBTQ with DCMU-pretreated samples [8].

Despite of the rather slow exchange rate of DCMU [44] there should still exist a nonzero probability of TBTQ interacting and covalently binding at an open site. Therefore a substitution of DCMU by TBTQ is anticipated to be detectable after a sufficiently long incubation time. This effect could explain the decrease of thermoluminescence emission in DCMU/5 min/TBTQ samples at increasing incubation time on ice (Fig. 6) provided that electrons stored on bound TBTQ give rise to only a low yield of excited singlet states after thermal detrapping and recombination with holes from the donor side. However, it has to be emphasized that these data cannot provide a proof because other parameters also affect the yield of singlets due to the detrapping and recombination process. This is shown by the mechanistically not yet clarified enhancement of the thermoluminescence upon increasing storage on ice of the TBTQ/5 min/DCMU-treated samples. Therefore more detailed investigations are required to analyze these phenomena.

On the basis of data presented in this study we conclude that TBTQ binding imposes a limited electron pool capacity at the PS II acceptor side that cannot be eliminated by DCMU (up to 100 μM). DCMU highly retards the regeneration of this pool and simultaneously accelerated the back reaction between the donor and acceptor side. The latter idea is supported by the disappearance of the O_2-yield at longer dark times between the flashes (Messinger, J., Hanssum, B. and Renger, G., unpublished results). The data of this study show that halogenated 1,4-benzoquinones provide a very interesting class of substances to analyze the interaction of quinones with the PS II complex.

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