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Stabilization of states I and II by *p*-benzoquinone treatment of intact cells of *Chlamydomonas reinhardtii*

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Intact cells of the unicellular green alga *C. reinhardtii* were treated with *p*-benzoquinone after adaptation to state I by far-red illumination or to state II by an incubation under anaerobic conditions. This reagent can be regarded both as an oxidant of the plastoquinones and a protein cross-linker. When algae placed in state I then treated with *p*-benzoquinone were transferred to state II conditions, and conversely, they retained the fluorescence yield characteristic of their initial state. We could thus obtain intact cells blocked in either state I or II with a similar oxidation state of the electron transfer chain. Analysis of the concentration dependences for state fixation by *p*-benzoquinone indicated that its reduction prevented state fixation, as expected from a cross-linking action mechanism. We present evidence for such a cross-linking action at the thylakoid membrane level. However, we could rule out the possibility that state fixation occurred through LHC immobilization due to an extensive protein cross-linking, since *p*-benzoquinone treatment did not prevent protein lateral diffusion along the thylakoid membranes. In contrast, it did prevent changes in LHC phosphorylation. We conclude that the inactivation of the enzymes (kinase and phosphatase) controlling LHC phosphorylation changes is responsible for state fixation. We discuss the mechanism of such enzyme inactivation by *p*-benzoquinone in terms of intramolecular cross-links.

Introduction

Higher plants and green algae undergo changes in light-energy distribution between the two photosystems which are associated with a change in phosphorylation of the main chlorophyll-protein complex of the PS II peripheral antenna (LHCII) (reviewed in Refs. 1–3). These extensive antenna changes may be accompanied by a change in the contribution of cyclic and linear electron flow to ATP production [4,5]. However, this possibility has been explored experimentally mainly with blue-green and red algae [6,7].

Changes in light-energy distribution were first described as 'state transitions' [8,9], a short-term chromatic adaption to light favouring either PS II or PS I excitation, such that algae developed an increased sensitisation of PS I (state II) or PS II (state I). Due to our improved knowledge on the control mechanism of state transitions, either of the two states can now be obtained independent of changes in light quality, in a variety of ways: by manipulating the redox state of the IEC [10], the ATP level in the chloroplast [11,12] or the availability in the ATP as a substrate for the LHC-kinase in experiments with isolated thylakoids [13]. However, the contrast in the fluorescence yields in states I and II varies widely, depending on the material and methods used to achieve the state transitions. Typical experiments with higher plant thylakoids yield 10–20% fluorescence quenching at room temperature in state II as compared to state I [14]. With intact algae, changes in light quality induced fluorescence changes of about the same amplitude, 10–20% [15], but more drastic changes in the redox states of the IEC, namely light plus DCMU (state I) versus anaerobiosis (state II), led to a 40–60% fluorescence quenching in state II as compared to state I [15,16]. Thus, the latter experimental situation is particularly suitable for a study of possible changes in the efficiencies of cyclic versus linear electron flows in the two states. However, a spectroscopic comparison of their electron transfer characteristics is hampered by the requirement of large differences in redox state of the

Abbreviations: IEC, intersystem electron carriers; p-BQ, *para*-benzoquinone; DCMU, dichlorophenyldimethylurea. STESR, saturation transfer electron spin resonance. MSL, *N*-proxylmaleimide; PAGE, polyacrylamide gel electrophoresis; LMC, light-harvesting complex; TAP, Tris-acetate/phosphate.

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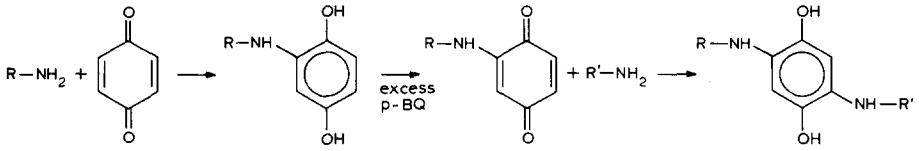

Fig. 1. Reaction scheme for protein cross-linking by p-BQ.

IEC, in order to maintain the algae is either of the two states. Moreover, the instability of state I, which rapidly reverts to an intermediate state in darkness, precludes any attempt to proceed to a reliable biochemical characterization of the changes in protein–protein interactions in the thylakoid membrane between the two states.

It is well documented that *p*-benzoquinone (p-BQ) oxidizes plastoquinones in intact algal cells [17,18]. p-BQ has also been used successfully as a protein cross-linker [19,20], reacting with their amino groups (Fig. 1). We show here that p-BQ treatment of intact algae placed in states I or II results in the fixation of the two states, thus providing an intact system placed in state I or II with an electron transfer chain in a similarly oxidised state.

Material and Methods

C. reinhardtii WT cells were grown in TAP medium under 300lx cool fluorescent light. Cells were harvested in mid-exponential phase ($4 \cdot 10^6$ cells/ml) and concentrated to 10^7 cells/ml in the same medium. State I conditions were 20 min preillumination under far-red light. State II conditions were 20 min incubation in darkness under nitrogen atmosphere. p-BQ treatment consisted of 5 min incubation with p-BQ in darkness (incubation in the light induced irreversible fluorescence quenching) followed by low-speed centrifugation ($200 \times g$ for 2 min) and resuspension in p-BQ-free TAP medium.

For reversion experiments, cells in state II, subsequently treated or not with p-BQ, were incubated for 20 min in darkness under vigorous stirring (state I conditions), whereas cells in state I, subsequently treated or not with p-BQ, were incubated for 20 min in darkness either with 2 mg/ml glucose oxidase and 20 mM glucose or under nitrogen atmosphere (state II conditions). Fluorescence induction experiments were then performed as in Ref. 21.

Similar experimental procedures were followed with cells labelled with [^{32}P]orthophosphate (2 μCi/ml) except that phosphate-free Tris-acetate (TA) medium was substituted for TAP medium. Analysis of ^{32}P-labeled membrane polypeptides was then carried out as in Ref. 15 except that $100\,000 \times g$ ultracentrifugations for 30 min substituted for any 15 min $50\,000 \times g$ centrifugation step in the conventional procedure for thylakoid membrane purification.

Mg^{2+}-induced fluorescence changes were studied using broken cell preparations as in Ref. 21. MSL-labelling of purified thylakoid membranes and STESR spectra were performed according to Ref. 22.

Results

Fig. 2 (left) shows a typical change in the fluorescence induction curves in presence of DCMU, between state I and state II. Whereas the fluorescence quenching at F_{max} was about 40% in state II vs. state I, the F_0 level in state II was much higher than in state I. The F_0 level in the presence of DCMU is, in part, controlled by the oxidation state of the secondary acceptor, Q_B [23]: owing to the equilibrium between the primary and secondary quinone acceptors, Q_A and Q_B, and to the higher affinity of DCMU for the oxidized Q_B pocket [24,25], binding of the inhibitor results in the closure of all PS II traps (state Q_A^-) initially connected with a secondary semiquinone anion (Q_B^-). Since extensive reduction of the plastoquinones occurred in the anaerobic state (state II), most of the PS II centres were closed upon DCMU addition, thus inducing a rise at FO.

In Fig. 2 (right) are shown the fluorescence induction curves of the same cells subsequently treated with p-BQ (for details, see Materials and Methods). Cells in state I, whether treated or not with p-BQ, showed the same fluorescence induction patterns. In contrast, the fluorescence yield of p-BQ-treated cells in state II, was now lower than in state I both at F_{max} and F_0. This was indicative of the oxidation by p-BQ of the semiquinone anions that had accumulated in state II. It is of note that the extensive quenching at F_{max} was still visible, despite this oxidising action of p-BQ. This indicated a stabilization of state II in oxidizing conditions. We note that the fluorescence quenching at F_0 was, however, lower than that F_{max}, which suggested that cation concentrations in the stroma of intact cells of *C. reinhardtii* may be low enough to elicit changes in spillover together with changes in antenna size during state transitions [26].

In order to further assess the ability of p-BQ to fix the algae in their initial state, we submitted p-BQ-treated algae in state I to state II conditions and conversely. To this end, we plotted on a fluorescence scale ranging from 0 (F_{max} in state II) to 100 (F_{max} in state I), the F_{max} level of algae first treated in either of the two states with variable concentrations of p-BQ then sub-

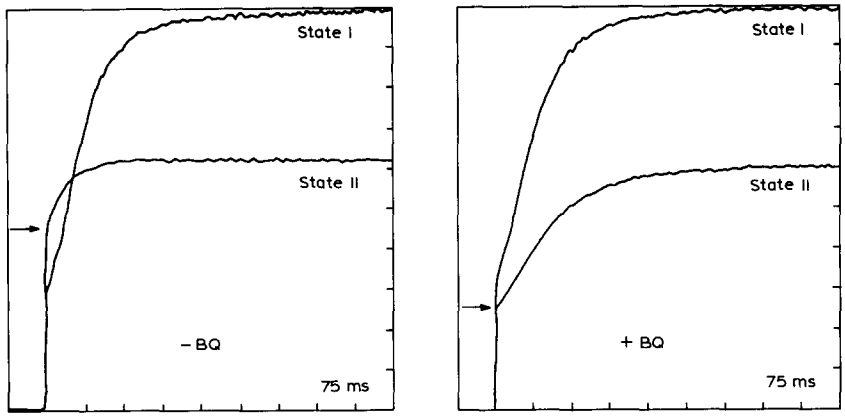

Fig. 2. Fluorescence induction curves, in the presence of DCMU, of algae placed in state I or in state II then (left) untreated or (right) treated with p-BQ.

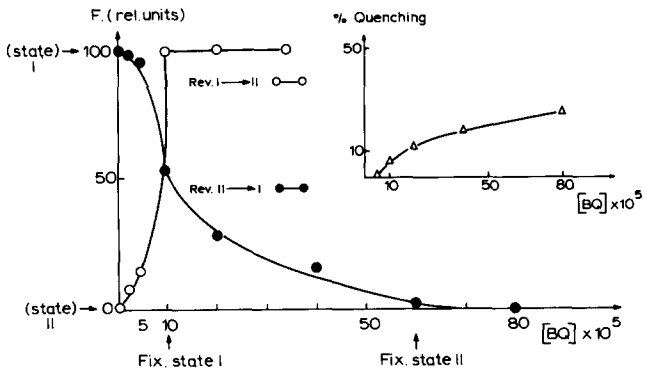

Fig. 3. Fixations of state I (open circles) and state II (dark circles) by increasing concentrations of p-BQ: algae were placed in state I or II then treated with p-BQ and placed in redox conditions corresponding to the opposite state. The maximum fluorescence yields, in the presence of DCMU, were plotted relative to that in state I (100) and that in state II (0) before p-BQ treatment. F_{max} values were corrected for chemical quenching by p-BQ. Inset: p-BQ-induced fluorescence quenching using algae placed in state I, then treated with p-BQ and incubated in darkness under vigorous stirring, for the same time as in the reversion experiments.

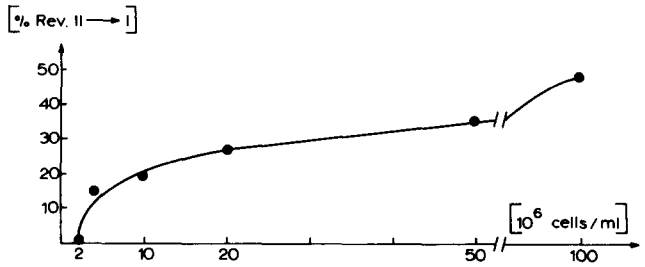

Fig. 4. Decreased efficiency of state II fixation by $3 \cdot 10^{-4}$ M p-BQ as a function of increasing cell concentrations. (%REV.II → I) corresponds to $(F_{max,i} - F_{max,\ state\ II})/(F_{max,\ state\ I} - F_{max,\ state\ II})$ where $F_{max,i}$ corresponds to the F_{max} of algae placed in state II at concentration C_i, then treated with p-BQ and placed in state I conditions.

jected to the opposite state condition, as a function of p-BQ concentration (Fig. 3). The F_{max} levels after p-BQ treatment and reversion were corrected for p-BQ quenching at each concentration (see inset Fig. 3).

Using cell suspensions at 10^7 cells/ml, we observed that state I was fixed with 10^{-4} p-BQ, whereas up to 6 10^{-4} M p-BQ was required to fix state II. In addition, the relative efficiency of state II fixation at a given p-BQ concentration depended strongly on cell concentration. This is illustrated on Fig. 4 by the decreasing efficiency of $3 \cdot 10^{-4}$ M p-BQ for state II fixation using algal suspensions from $2 \cdot 10^6$ to $1 \cdot 10^8$ cells/ml. Thus, the more reducing power brought about by the cells, the higher was the p-BQ concentration required for state fixation. This is consistent with the cross-linking mechanism described in Fig. 1, which involved only p-BQ and not its reduced forms.

We then investigated the mechanism of state fixation by p-BQ. We first examined whether LHC displacement along the thylakoid membranes could be prevented due to extensive protein cross-linking by p-BQ. In Table I are gathered some of the thylakoid membrane modifications induced by p-BQ treatment. A most striking change occurred in the mechanical properties of the thylakoid membranes upon French press disruption. Whereas thylakoid membrane vesicles from untreated cells broken in destacking conditions were found in a 50000 × *g* 15 min pellet, the majority of the thylakoid vesicles from

TABLE I

Evidence for p-BQ cross-linking action on thylakoid membranes

	% Chl in sup. (50000 × *g*, 15 min) [b]	τ app.(s) [c]	protein aggregates upon SDS-PAGE
−p-BQ	<10	$1 \cdot 10^{-5}$	−
+p-BQ [a]	60	$8 \cdot 10^{-5}$	+

[a] Algae in state I were incubated 5 min with $2 \cdot 10^{-4}$ M p-BQ.

[b] Recovered after breaking intact cells through a French press in destacking conditions.

[c] τ_c values from the second harmonic spectra are obtained as in Ref. 22.

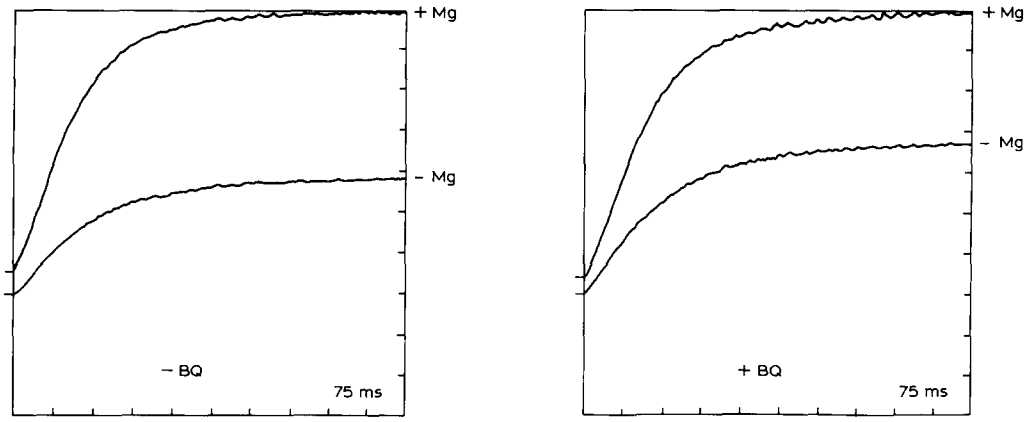

Fig. 5. Left: Mg^{2+}-induced fluorescence changes on algae broken through a Yieda press in the absence of Mg^{2+}. Right: same experiments with algae treated with p-BQ before breaking.

p-BQ-treated cells remained in the supernatant. These smaller vesicles were pelleted only at $100\,000 \times g$ 30 min.

The decrease in the vesicle sizes suggested important modifications of the structure of the thylakoid membranes. This was further investigated by studying the protein rotational mobility in the thylakoid membranes. Saturation transfer electron spin resonance (STESR) of thylakoid membranes labelled with a paramagnetic analogue of *N*-ethylmaleimide (MSL), were previously shown to label mainly LHC and PS I protein subunits

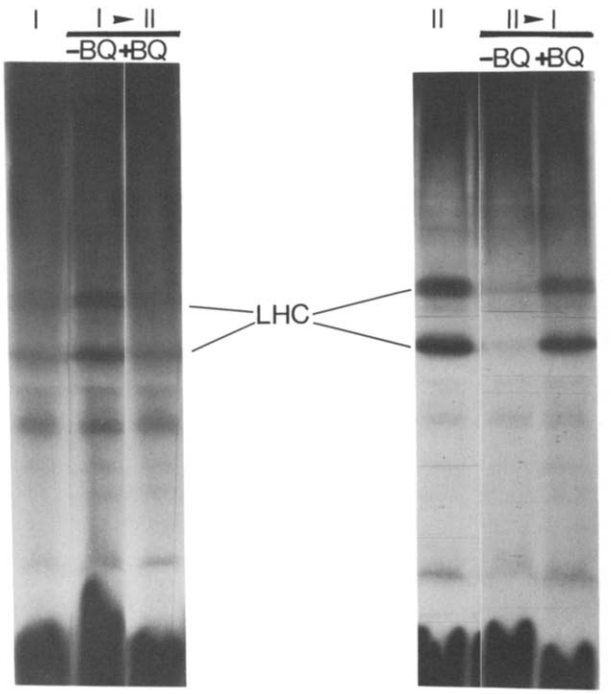

Fig. 6. Autoradiogram of ^{32}P-labelled polypeptides after SDS-PAGE of purified thylakoid membranes. Cells were incubated with [^{32}P]P_i then placed in state I (left) or in state II (right). They were subsequently treated or not with p-BQ and placed in state II conditions (left) on in state I conditions (right). All samples were then treated with p-BQ before thylakoid membrane purification.

[22]. Thylakoid membranes originating from cells in state I treated with 10^{-4} M p-BQ displayed a 5–10-times lower rotational mobility, as compared to non-treated samples (Table I). A limited number of MSL-labelled proteins were susceptible to p-BQ immobilization, since no further increase in rotational correlation time was observed upon raising the p-BQ concentration to 10^{-3} M with thylakoid membranes isolated from 10^{-4} M p-BQ-treated cells (results not shown). On the other hand, SDS-PAGE of thylakoid membrane proteins from p-BQ-treated cells resolved the same polypeptides, in similar relative amounts, as with untreated cells. However some aggregates, which did not enter the stacking gel (5% acrylamide), were observed with p-BQ-treated membranes (Table I). Thus, both the STESR and SDS-PAGE experiments were consistent with a limited but significant action of p-BQ as an intermolecular cross-linker.

We then assessed the lateral mobility of thylakoid membrane proteins in p-BQ-treated cells. To this end, cells were placed in state I, then treated with p-BQ (from $2 \cdot 10^{-4}$ to $6 \cdot 10^{-4}$M), broken in a Yieda Press and washed in destacking conditions [21]. Mg^{2+}-induced fluorescence changes were then analysed by adding 10 mM $MgCl_2$ to the resuspension medium. Fig. 5 shows that Mg^{2+}-induced fluorescence changes were similar, whether the algae were treated or not with p-BQ. Similarly, untreated cells, broken in destacking conditions then treated with p-BQ still displayed an increase at F_{max} upon $MgCl_2$ addition (not shown). These experiments demonstrated that changes in spillover, which correspond to changes in the lateral distribution of PS II vs. PS I due to membrane restacking, still occurred at p-BQ concentrations which were above those required for fixation of state I. State fixation due to an immobilisation of LHC next to either PS II or PS I was therefore unlikely.

Fig. 6 shows the phosphorylation status of LHC subunits from cells treated or not with p-BQ in one

state then placed in the opposite state conditions. Suitable concentrations for state I and state II fixation were chosen from the fluorescence experiments in Fig. 3. We observed that p-BQ treatment prevented further changes in the LHC phosphorylation: cells blocked in state I retained a dephosphorylated LHC even in state II conditions, whereas cells blocked in state II retained a phosphorylated LHC even in state I conditions. Thus, p-BQ treatment blocked some steps in the mechanism controlling LHC-phosphorylation changes.

Discussion

Although we did not observe any significant restriction of the lateral diffusion of the thylakoid membrane proteins (Fig. 5), our STESR study showed that some specific protein–protein interactions may be stabilised by p-BQ. However, since the LHC phosphorylation status became invariant after p-BQ treatment, it is likely that state fixation by p-BQ occurred through its action on the kinase/phosphatase system, probably by intramolecular cross-links. A selective inhibition by p-BQ of several enzymes, like the ATP synthase or flavoproteins, has been previously reported [18,27–29]. A common feature of these enzymes is to have (poly)nucleotide binding sites. Interestingly, such enzymes can be purified by affinity chromatography with Blue dextran Sepharose. Their affinity has been attributed to the recognition of a dinucleotide fold typical of several ATP and NAD(P)-binding sites [30] by Cibacron blue, which is an anthraquinone dye [31]. Indeed, spinach CF1 shows a significant affinity for a large family of anthraquinone derivatives [32]. Therefore, a possible mechanism for p-BQ action is the recognition of the conserved topologies in nucleotide binding proteins, as would be the case with the p-BQ ring of anthraquinones. Subsequent intramolecular cross-links with ϵ-NH_2 lysines located in the dinucleotide fold [30] may then allow an irreversible inactivation of the enzymes by p-BQ. Such a mechanism, suitable for an inactivation of the LHC-kinase which binds ATP, raises the question of the possible existence of nucleotide binding sites on the thylakoid-bound phosphatase which is responsible for LHC dephosphorylation. Control of state transitions by changes in the ATP concentration in the chloroplast [11,12] may then occur through an ATP- or ADP-mediated regulation of the phosphatase activity.

Since no alteration in photosynthetic electron transfer occurs in p-BQ-treated cells [17,18,28], this treatment provides a unique cellular material to study photosynthetic activities in states I and II. In addition, it provides an improved starting material for biochemical studies of the changes in protein/protein interactions after solubilization of thylakoid membranes. Using such p-BQ-treated cells, we have recently obtained preliminary evidence for a state-dependent change in the interaction of cytochrome b_6/f complexes with PS I and PS II reaction centres [33].

Acknowledgements

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