

Redox-State Dependent Changes of Inhibitor-Binding to the Photosystem II Acceptor Complex

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Binding of radioactively labelled DCMU, ioxynil and terbutryn to spinach chloroplasts is determined following preillumination by single-turnover, saturating light flashes. With all of the three herbicides binary oscillations of binding are observed. Dark-adapted samples, or those preilluminated by an even number of flashes, bind more inhibitor than samples preilluminated by an odd number of flashes. Binding oscillations depend on inhibitor incubation time and on the seasonal adaptation of the plants. The binding kinetics following a single flash display three phases, the last two of which can be correlated with reoxidation kinetics of the secondary photosystem II acceptor Q_B^- , as determined by fluorescence measurements. Analysis of the binding at DCMU concentrations up to 10^{-7} M free DCMU yields identical binding constants for dark- and flash preilluminated samples, but much less binding sites following one flash. It is concluded that up to 10^{-7} M free DCMU, centers with bound Q_B^- do not contribute significantly to total binding. Displacement of Q_B^- from the binding site is half-saturated at about 10^{-6} M DCMU, as monitored via fluorescence induction. The data are considered strong support for the Velthuys 'inhibitor- Q_B^- competition model'.

Introduction

Many studies on the photosystem II acceptor complex suggest that electron transport from the primary acceptor Q_A to the pool of plastoquinone proceeds via a secondary acceptor Q_B , previously called 'B' or 'R' [1–6]. Q_B is able to store one electron until upon a second photoact two electrons are accumulated and then released into the plastoquinone pool. By the use of inhibitors like DCMU which block reoxidation of Q_A and by application of single-turnover light flashes a careful investigation of this two electron gating mechanism at the photosystem II acceptor side has been possible. Observations of binary oscillations in DCMU-induced fluorescence increase, in dependence of the number of preilluminating, single-turnover flashes, has been considered a strong argument in favour of this concept [1, 3, 4].

Recently Velthuys proposed that inhibitor binding at the PS II acceptor complex is in competition with the binding of plastoquinone [7]. In this model, Q_B^- is a plastosemiquinone anion which becomes stabilized by binding to the primary acceptor Q_A . Inhibitor binding can take place only when the binding site is vacant, *i.e.* when not occupied by Q_B^- . Support for this model has come from fluorescence studies [6, 8, 9] and, more directly, from [14 C]DCMU binding studies [10, 11].

Here, we report in more detail on redox-state dependent binding of radioactively labelled PS II inhibitors. Particular attention is given to a population of PS II centers which in previous studies appeared to bind DCMU in a redox independent manner [10, 11]. It will be shown, that a substantial part of Q_B^- is reoxidized following a single flash by a yet unknown mechanism and, therefore, will display the same binding properties as dark adapted centers.

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; K_B , equilibrium constant of inhibitor binding; MES, 2-(N-morpholino)-ethanesulfonic acid; terbutryn, 2-(tert-butylamino)-4-(ethylamino)-6-(methylthio)-s-triazine; PS II, photosystem II; Q_A , primary quinone electron acceptor of photosystem II; Q_B , secondary quinone electron acceptor.

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Materials and Methods

All experiments were carried out with isolated chloroplast membranes from greenhouse-grown *Spinacia oleracea*. The isolation of the membranes was performed as described in [12] with the modifications as in [10]. The preparation of intact chloroplasts was stored in the dark on ice for at least 3 h. Intact chloroplasts were osmotically shocked in a



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medium containing 50 mM MES (KOH) buffer, pH 6.5, and 5 mM MgCl_2 . After addition of the same volume of a medium containing 0.66 M sorbitol and the same concentration of buffer (pH 6.5) and MgCl_2 as the shock medium, 10^{-4} M $\text{K}_3\text{Fe}(\text{CN})_6$ were added. 5 min later, the thylakoids were incubated with 5×10^{-3} M NH_2OH for at least 15 min to prevent reoxidation of Q_A^- via the backreaction [13]. For binding experiments the following radioactively labelled inhibitors were used: [^{14}C]DCMU (34.4 $\mu\text{Ci}/\text{mg}$), [^{14}C]terbutryn, a *s*-triazine (7.9 $\mu\text{Ci}/\text{mg}$) and [^{14}C]ioxylnil, a benzonitrile (34.3 $\mu\text{Ci}/\text{mg}$).

Saturating light flashes of about 5 μs duration were applied at 1 Hz with a Strobotac Xenon flash tube (General Radio) by focussing the light into the reaction vials. Herbicides were dissolved in methanol and were added to the assays always under vigorous stirring. The final methanol concentration never exceeded 1% (v/v). After the given incubation times the samples were centrifuged (Beckman Mini-fuge B) at about $9000 \times g$ for 15 s. Within 5 s about 90% of the thylakoids were in the pellet. Aliquots of the clear supernatant were added to scintillation fluid (Zinsser, Quickszint 2000) and counted for radioactivity in a liquid scintillation counter (Contron Betamatic). Each sample was corrected for quenching. DCMU-induced changes of chlorophyll fluorescence were measured as in [10]. Even in the presence of DCMU and of NH_2OH the measuring beam was weak enough ($10^{-4} \text{ W} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) not to cause any fluorescence increase. In all experiments, the temperature was kept at 10 °C.

Results and Discussion

1. Flash-induced binary oscillations of herbicide binding

Binary oscillations in the amount of binding of PS II herbicides to thylakoid membranes are shown in Fig. 1. Addition of the inhibitors took place 10 s after the last flash and samples were incubated for 5 min with the inhibitor before binding was stopped by centrifugation. In dependence of the number of preilluminating flashes, strong binding was found after zero or an even number and weak binding after an odd number of flashes. The amplitude of the oscillations varied between 30 and 60% of the maximum binding in the dark and was damping out with increasing number of flashes. The occurrence

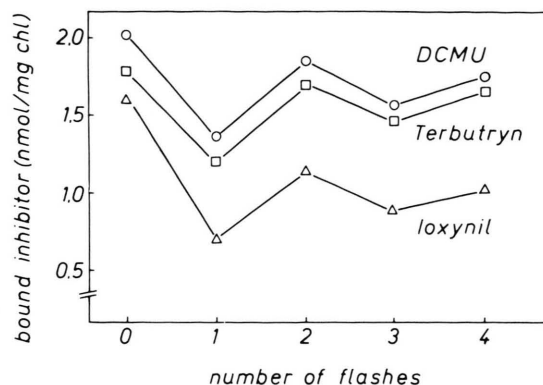


Fig. 1. Binding of [^{14}C]DCMU (\circ), [^{14}C]terbutryn (\square) and of [^{14}C]ioxylnil (\triangle) at pH 6.5, in dependence of the number of preilluminating flashes. Total inhibitor concentration 2×10^{-7} M, incubation time 5 min, inhibitor added 10 s following the last flash, Chl, 25 $\mu\text{g}/\text{ml}$.

of oscillations in inhibitor binding strongly suggests a decisive influence of the redox-state of the charge accumulating secondary acceptor Q_B . With zero or an even number of flashes Q_B is oxidized, while with one or an odd number of flashes Q_B is singly reduced. The fact that the DCMU-type inhibitor terbutryn as well as the phenol-type inhibitor ioxylnil also display binary oscillations in binding supports previous conclusions on a common binding site [14, 15].

2. Redox-state dependent binding kinetics

In evaluating the results of Fig. 1 it is important to note that inhibitor binding was assayed after 5 min incubation period, which is sufficient to reach a quasi-stationary level of binding, following a single flash. The actual time dependency of binding is shown in Fig. 2. Under the given conditions, three phases of DCMU-binding following a single flash can be distinguished: A rapid phase (phase 1) occurring within less than 10 s, a slower phase (phase 2) with a half-time of about 1 min and a very slow phase (phase 3) with less than 10% increase of total binding within 45 min. Dark-adapted samples reach equilibrium within less than 20 s. As a consequence of these binding kinetics, the amplitude of the redox-state dependent binding oscillations depends on incubation time, with largest amplitudes after short times.

Comparing the data of Figs. 1 and 2 with previously reported results from our laboratory [10, 11], there are significant differences in the relative

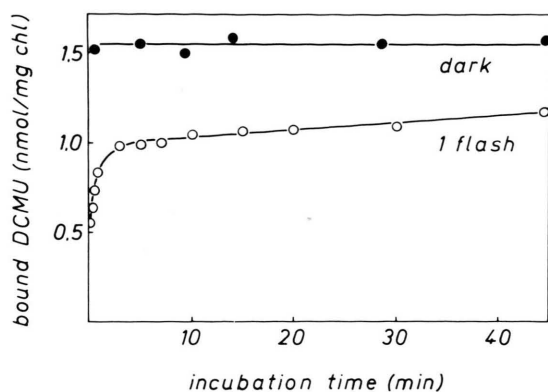


Fig. 2. Kinetics of [^{14}C]DCMU binding in the dark (●) and after one preilluminating flash (○). Inhibitor addition 15 s after the flash, total [^{14}C]DCMU concentration 1.5×10^{-7} M, pH 6.5, Chl, 20 $\mu\text{g}/\text{ml}$.

extents of the three binding phases following a single flash. While these differences are by far too large to be caused by variation of experimental parameters, there seems to be a distinct effect of seasonal adaptation of spinach plants. As it appears, there is a tendency for a large 'phase 1' and a small 'phase 3' in winter spinach [10]. On the contrary, summer spinach shows a small 'phase 1' and a large 'phase 3' [11].

3. Stability of the semiquinone anion Q_B^-

According to a simple model of charge accumulation at the PS II acceptor side, a single-turnover, saturating flash should produce almost quantitatively Q_B^- , provided the acceptor complex is completely oxidized before the flash. Furthermore this Q_B^- should be very stable, if reoxidation via the PS II back-reaction is prevented by NH_2OH . The data of Fig. 2 and of previous reports [10, 11] cannot agree with such a simple model, when at the same time the 'inhibitor-plastoquinone competition model' shall apply, *i.e.* that DCMU will bind only to centers without bound Q_B^- . Obviously even after a single flash a considerable amount of DCMU can be bound (see Fig. 2), *i.e.* a considerable degree of Q_B^- reoxidation would have to take place by mechanisms which are 'forbidden' by a simple charge-accumulation model.

Reoxidation of Q_B^- can be monitored by measuring the DCMU-induced rise in dark fluorescence in dependence of the time following a flash. Under conditions which were almost identical to those of

the [^{14}C]DCMU binding experiments, such fluorescence measurements yielded the reoxidation kinetics depicted in Fig. 3. As it appears, part of Q_B^- becomes reoxidized with a half-time of about 1 min, while another part (approximately 50%) stays reduced even for extended dark-times.

These fluorescence data suggest a marked heterogeneity of PS II centers with respect to their capability of reoxidizing Q_B^- by a mechanism different from charge accumulation.

4. Analysis of concentration dependent inhibitor binding

In Fig. 4 the concentration dependency of [^{14}C]DCMU binding with and without flash preillumination, and in Fig. 5 the corresponding double reciprocal plots are depicted. Under our experimental conditions, with and without flash preillumination, high-affinity binding saturated at about 5×10^{-8} M free DCMU. The double reciprocal plot suggests identical binding constants of 1.8×10^{-8} M, while the number of binding sites was cut down to about half by a preilluminating flash.

Following a formal analysis according to enzyme kinetics one may be led to conclude that preillumination by a single flash results in non-competitive binding of inhibitor and Q_B^- (the latter created in

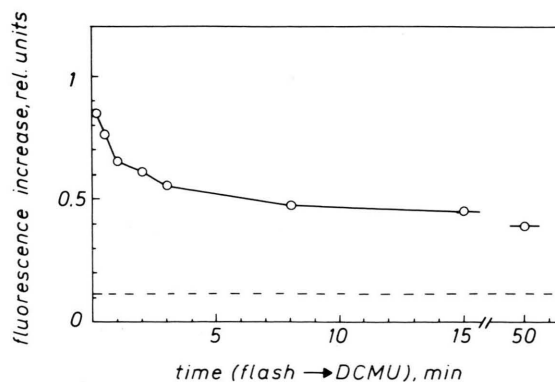


Fig. 3. DCMU-induced increase of chlorophyll fluorescence in dependence of the time between a saturating, single-turnover flash and DCMU-addition. Sample-preincubation 15 min in the dark in presence of 5 mM NH_2OH and 0.1 mM $\text{K}_3\text{Fe}(\text{CN})_6$. T , 10 $^\circ\text{C}$, pH 6.5, Chl, 7.5 $\mu\text{g}/\text{ml}$. Final DCMU concentration 10^{-5} M. The increase of fluorescence 30 s following injection is plotted. One relative unit corresponds to the dark fluorescence level before application of a flash. The dotted line gives the level of DCMU-induced fluorescence rise without preilluminating flash.

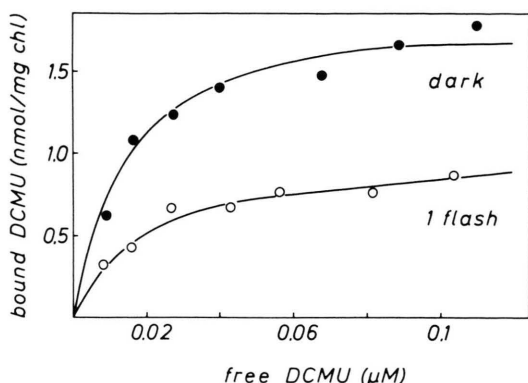


Fig. 4. Concentration dependent binding of $[^{14}\text{C}]$ DCMU to thylakoids, dark adapted (●) or flash preilluminated (○). DCMU incubation time 5 min, pH 6.5, Chl, 25 $\mu\text{g}/\text{ml}$.

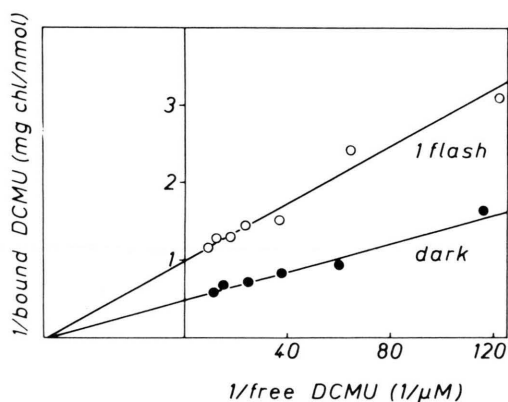


Fig. 5. Double reciprocal plot of concentration dependent $[^{14}\text{C}]$ DCMU binding to thylakoids, dark adapted (●) or flash preilluminated (○). The abscissa intercepts reveal K_b , the ordinate intercepts the concentration of binding sites.

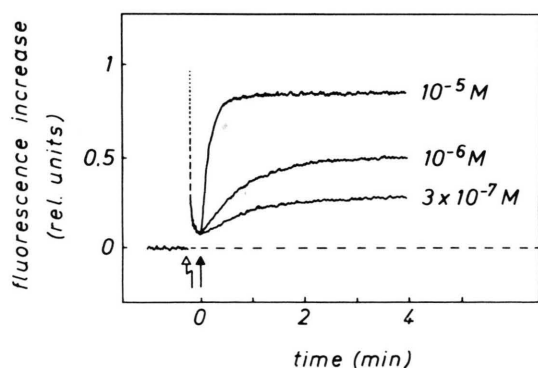


Fig. 6. DCMU-induced fluorescence increase following a single, saturating flash in dependence of DCMU-concentration. DCMU-injection at time zero, 15 s following a flash. T , 10 $^{\circ}\text{C}$, pH 6.5, Chl, 20 $\mu\text{g}/\text{ml}$. One relative unit corresponds to the dark fluorescence level before flash-illumination.

the flash), to two separated receptor sites. Such approach has proven useful in a large number of investigations on the mode of competition of different herbicides [14–17]. In the present case, however, such conclusion would be in contradiction to the original 'inhibitor- Q_{B}^{-} competition model' [7] as already pointed out previously [11]. Our new observation of partial Q_{B}^{-} reoxidation following a single flash (see Fig. 3) may resolve this apparent contradiction: Under the given conditions, even following a single flash, at about half of all centers the binding site is vacant from Q_{B}^{-} during the incubation period of the inhibitor. Hence, the inhibitor binding properties displayed in Figs. 4 and 5 appear to reflect only those centers with vacant binding sites, with as well as without flash preillumination. This explains the identical K_b -values. Within the used DCMU concentration range (up to $3 \times 10^{-7}\text{ M}$) and under the given conditions, displacement of Q_{B}^{-} by the inhibitor seems to play a minor role as compared to binding to vacant sites. Measurements of the DCMU-induced fluorescence increase following a single flash confirms that, at pH 6.5 and 10 $^{\circ}\text{C}$, displacement of Q_{B}^{-} by $3 \times 10^{-7}\text{ M}$ DCMU is rather slow ($t_{1/2} \cong 1\text{ min}$) and incomplete (about 30% within 4 min incubation) (see Fig. 6). It was previously shown by Laverne [8] that half-saturation of DCMU-induced fluorescence increase is at a concentration of $6.5 \times 10^{-7}\text{ M}$ at pH 7.

Discussion

The presented binding data, in combination with the fluorescence data give strong further support to the 'inhibitor-plastoquinone competition model' proposed by Velthuys [7] and elaborated by Laverne [8, 9]. The data suggest that the singly reduced, secondary PS II acceptor Q_{B}^{-} binds strongly to the inhibitor binding site and thus prevents significant inhibitor binding within a concentration range (up to about 10^{-7} M free DCMU) which gives saturated binding at oxidized centers (see Figs. 4 and 5). Displacement of Q_{B}^{-} by DCMU, which causes a fluorescence increase by electron reversal on the primary acceptor Q_{A} , will occur at considerably higher inhibitor concentrations (see Fig. 6 and [9]) and therefore, does not contribute significantly to binding properties displayed in Figs. 4 and 5. Binding experiments with higher inhibitor concentrations proved to be rather difficult because of the unfavourable ratio of bound to free $[^{14}\text{C}]$ DCMU.

There is a considerable amount of inhibitor binding following a single flash which at first sight appears to be redox-state independent and actually has been interpreted that way [10]. However, the fluorescence data of Fig. 3 suggest that under conditions of binding experiments, there is substantial reoxidation of Q_B^- following a flash by a yet unknown mechanism. As this reoxidation can take place before and during inhibitor incubation, DCMU can bind to the thus vacated binding site. Such explanation appears most reasonable to account for 'phase 2' and 'phase 3' of the DCMU binding kinetics displayed in Fig. 2. There remains uncertainty about 'phase 1'. For times shorter than 10 s it is difficult to determine Q_B^- by measuring the DCMU-induced fluorescence increase, as this increase is then overlapping with the fairly steep relaxation kinetics of the flash-induced fluorescence increase.

The question arises by which mechanism Q_B^- becomes reoxidized following a single flash. As shown in Fig. 3, this reoxidation is not due to the presence of a significant amount of Q_B^- before the flash. Also, the presence of 5 mM NH_2OH should block charge recombination at the PS II centers and prevent double-hits [18]. An outstanding feature of this type

of Q_B^- reoxidation appears to be its large variability with seasonal adaptation of the plants. One may consider the possible existence of an additional redox component in the PS II acceptor complex, which may either feed an electron into Q_B^- or receive an electron from it. Both cases would lead to reoxidation of Q_B^- and to its release from the binding site. The seasonal fluctuations of the amount of DCMU bound following a single flash may indicate variability in the relative concentration of such hypothetical, alternate acceptor or of its reduction level. There are numerous reports from Joliot's laboratory [19–21] and by Lavergne [9, 22, 23] on alternate PS II acceptors. Also, the 'high potential' PS II acceptors described by Bowes *et al.* [24] and by Hardt [25] may be involved.

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- [1] B. R. Velthuys and J. Ames, *Biochim. Biophys. Acta* **333**, 85–94 (1974).
- [2] B. Bouges-Bocquet, *Biochim. Biophys. Acta* **314**, 250–256 (1973).
- [3] F.-A. Wollmann, *Biochim. Biophys. Acta* **503**, 263–273 (1978).
- [4] J. Lavergne and A.-L. Etienne, *Biochim. Biophys. Acta* **593**, 136–148 (1980).
- [5] J. M. Bowes and A. R. Crofts, *Biochim. Biophys. Acta* **590**, 373–384 (1980).
- [6] H. H. Robinson and A. R. Crofts, *FEBS Lett.* **153**, 221–226 (1983).
- [7] B. R. Velthuys, *FEBS Lett.* **126**, 277–281 (1981).
- [8] J. Lavergne, *Biochim. Biophys. Acta* **679**, 12–18 (1982).
- [9] J. Lavergne, *Biochim. Biophys. Acta* **682**, 345–353 (1982).
- [10] H. Laasch, W. Urbach, and U. Schreiber, *FEBS Lett.* **159**, 275–279 (1983).
- [11] H. Laasch, U. Schreiber, and W. Urbach, *Proc. 6th Intern. Congress on Photosynthesis*, Brussels, 1983, in press.
- [12] W. Cockburn, D. A. Walker, and C. W. Baldry, *Plant Physiol.* **43**, 1415–1418 (1968).
- [13] P. Bennoun, *Biochim. Biophys. Acta* **216**, 357–363 (1970).
- [14] H. Laasch, K. Pfister, and W. Urbach, *Z. Naturforsch.* **37c**, 620–631 (1982).
- [15] C. J. van Assche and P. M. Carles, *Biochemical responses induced by herbicides* (M. J. Comstock, ed.), ACS Symposium Series, 1–21 (1982).
- [16] W. Oettmeier and K. Masson, *Pest. Biochem. Physiol.* **14**, 86–97 (1980).
- [17] W. Tischer and H. Strotmann, *Biochim. Biophys. Acta* **460**, 113–125 (1977).
- [18] G. A. den Haan, H. Gorter de Vries, and L. N. M. Duysens, *Biochim. Biophys. Acta* **430**, 265–281 (1976).
- [19] P. Joliot and A. Joliot, *Biochim. Biophys. Acta* **546**, 93–105 (1979).
- [20] P. Joliot and A. Joliot, *Proc. 5th Intern. Congress on Photosynthesis* (G. Akoyunoglou, ed.), **Vol. III**, 885–899, Balaban Int. Sci. Serv., Philadelphia 1980.
- [21] P. Joliot and A. Joliot, *FEBS Lett.* **134**, 155–158 (1981).
- [22] J. Lavergne, *Photobiochem. Photobiophys.* **3**, 257–271 (1982).
- [23] J. Lavergne, *Photobiochem. Photobiophys.* **3**, 273–285 (1982).
- [24] J. M. Bowes, A. R. Crofts, and S. Itoh, *Biochim. Biophys. Acta* **547**, 320–335 (1979).
- [25] H. Hardt, *Biochim. Biophys. Acta* **635**, 631–644 (1981).