Binary flash-induced oscillations of [^{14}C]DCMU binding to the photosystem II acceptor complex

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Binding of radioactively-labelled DCMU to spinach chloroplast membranes is determined following preillumination by single turnover saturating light flashes. When binding is assayed rapidly following inhibitor addition (<40 s) distinct binary oscillations of [^{14}C]DCMU binding can be observed. By dark-adapted samples or those, following an even number of flashes, more inhibitor is bound than after one or an odd number of flashes. During longer incubation times (>2 min), [^{14}C]DCMU binding becomes independent of flash preillumination. Comparison of the [^{14}C]DCMU binding kinetics following one flash and the kinetcis of the DCMU-induced increase of chlorophyll fluorescence rise reveals a substantial amount of binding which is not accompanied by a corresponding fluorescence rise. These data are discussed within the framework of the 'inhibitor-plastoquinone competition model' [FEBS Lett. (1981) 126, 277–281] and with reference to DCMU binding data derived from fluorescence measurements [Biochim. Biophys. Acta (1982) 682, 245–253].

DCMU-binding *Binary oscillation* *Chlorophyll fluorescence* *Inhibitor binding kinetics*

1. INTRODUCTION

Photosystem II (PS II) inhibitors of the DCMU-type block reoxidation of the primary acceptor Q [1]. Up to 3 nmol bound DCMU/mg chl, inhibitor binding takes place only at the specific receptor site of the PS II acceptor complex [2,3]. Upon DCMU addition there is an increase of fluorescence yield in the dark [4]. Its amplitude displays binary oscillations depending on the number of preilluminating flashes [5,6]. These oscillations are an expression of a two electron gating mechanism between Q (one electron carrier) and the pool of plastoquinone (two electron carrier). A secondary quinone acceptor, called R [5] or B [7] was postulated to store one electron until upon a second charge separation at the same center, the accumulated two electrons are released into the pool. It was originally proposed [5], that DCMU acts by lowering the midpoint potential of the secondary acceptor, thus shifting the equilibrium ($Q^- \cdot R \rightleftarrows Q \cdot R^-$) to the left.

Velthuys has proposed a modification of his original charge accumulation model, which has particular consequences for the interpretation of the mode of action of DCMU-type inhibitors [8,9]. In the new model, R is no longer a fixed molecule, but rather an ordinary plastoquinone molecule freely exchangeable with the pool as long as it is oxidized. However, upon formation of the semiquinone, this is supposed to be stabilized by binding to the primary acceptor site. It was suggested [8,9] that DCMU-type inhibitors act by competing with plastoquinone for a common binding site. Hence, inhibitor binding should be possible only if the binding site is vacant, i.e., if it is not occupied by the semiquinone. This new model has already found substantial support from fluorescence studies [10–12].

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *n*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid

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Here, we report on binding of radioactively labelled DCMU in dependence of the redox state of the acceptor complex. As predicted by the model, [^{14}C]DCMU binding shows binary oscillations depending on the number of preilluminating flashes, providing binding is assayed rapidly following addition of the inhibitor. However, comparison of the [^{14}C]DCMU binding kinetics and the kinetics of the DCMU-induced fluorescence increase following one flash reveals a substantial amount of rapid inhibitor binding which is not accompanied by a fluorescence rise.

2. MATERIALS AND METHODS

Intact chloroplasts were isolated from freshly harvested spinach leaves as in [13] with the following modifications: The leaves were homogenized in a medium containing 0.33 M sorbitol, 50 mM MES/KOH (pH 6.5) and 5 mM $MgCl_2$. After the first centrifugation of the homogenate, chloroplasts were transfered into a medium containing 0.33 M sorbitol, 50 mM HEPES/KOH (pH 7.5) and 5 mM $MgCl_2$. The chloroplasts were stored in the dark at 0°C for at least 5 h, to synchronize all reaction centers in the oxidized state. Aliquots of intact chloroplasts were ruptured by diluting in hypotonic medium (5 mM $MgCl_2$ and 10 mM MES/KOH (pH 6) or 10 mM HEPES for pH 7.5) and then resuspended isotonically by addition of an equal amount of the following medium: 0.66 M sorbitol and 5 mM $MgCl_2$, 90 mM MES/KOH (pH 6) or 90 mM HEPES/KOH, pH 7.5). The reaction medium also contained 50 μM $K_3^-(FeCN)_6$, to assure full oxidation of the PS II reaction centers, and 50 mM NH_2OH, to prevent reoxidation of Q^- via the backreaction [14].

The experimental procedure was as follows: After osmotic rupture and isotonical resuspension, $K_3(FeCN)_6$ and NH_2OH were added to aliquots (1 ml) of the thylakoid suspension contained in transparant centrifugation vials at 10°C. Saturating light flashes of about 10 μs duration were applied at 1 Hz with a General Electics FT 230 Xenon flash tube. Radioactively labelled [^{14}C]DCMU (34.4 μCi/mg) was added with vigorous stirring 10 s after the last flash. After further 10 s, the samples were centrifuged at $9000 \times g$ in a Beckman Minifuge B for 15 s. It was checked that within 5 s about 90% of the thylakoids were in the pellet. Aliquots 0.4 ml of the clear supernatant were added to 3 ml scintillation fluid (Roth, Rotiscint 33) and counted for radioactivity in a Contron Betamatic scintillation counter. Each sample was corrected for quenching. For further details on the binding experiments see [15].

DCMU-induced changes of chlorophyll fluorescence were measured as in [16]. Even in the presence of DCMU and NH_2OH the measuring beam was weak enough (10^{-4} W . m^{-2} . s^{-1}) not to cause any fluorescence increase. As with the binding experiments, temperature was kept constant at 10°C.

3. RESULTS AND DISCUSSION

3.1. *Binding experiments*

In fig. 1, the binding of [^{14}C]DCMU in dependence of the number of preilluminating flashes is shown. Inhibitor binding displays binary oscillations, with stronger binding following zero or an even number of flashes and weaker binding after an odd number of flashes. The oscillation amplitude amounts to about 20% of the total binding and is damping out with an increasing number of preilluminating flashes.

Two conditions were found essential to readily observe the binding oscillations displayed in fig. 1:

(1) Following addition of [^{14}C]DCMU the thylakoids have to be rapidly separated from the supernatant. With increasing times between inhibitor addition and centrifugation, the oscillation amplitudes descreases.

(2) A low pH is favourable for pronounced oscillations. At pH 7.5, the oscillation amplitude is

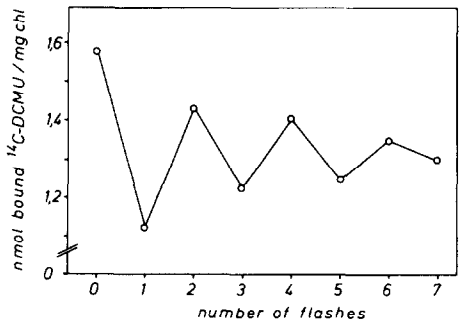

Fig. 1. Binding of [^{14}C]DCMU to thylakoids at pH 6 in dependence of the number of preilluminating light flashes; total [^{14}C]DCMU conc., 10^{-7}M; chl., 50 μg/ml.

only 10% and already fully damped after 3 flashes (not shown).

The importance of the incubation time and of the pH suggested a link between the inhibitor binding kinetics and the binary oscillations. As already concluded [11] from fluorescence experiments, DMCU binding is substantially slowed down by a preilluminating flash and by low pH conditions. At pH 6 [^{14}C]DCMU binding to dark-adapted thylakoids is completed within 15 s, while following a single flash only about 70% of the maximum binding in the dark is reached 15 s after inhibitor addition (fig. 2). The remaining amount of 30% took about 2 min to be completely bound. Interestingly, under equilibrium conditions the same amount of inhibitor is bound by the preilluminated as by the dark-adapted sample. In other experiments sometimes the amount of DCMU bound in a one-flash preilluminated sample was smaller than that in a dark-adapted sample, but never 10% of the maximum binding. Hence, to observe optimal oscillations which reflect redox state dependent differences in DCMU binding properties, it is essential to stop inhibitor binding at an early time after mixing. Also, low pH conditions facilitate a stop of inhibitor binding at an early stage of the process by slowing down the binding kinetics.

These findings can be partially accounted for by the new Velthuys model [8,9] of competition between inhibitor (I) and plastoquinone (PQ) for a common binding site at the PS II reaction center complex:

$$Q \cdot PQ^- \rightleftarrows Q^- \cdot PQ \rightleftarrows Q^- + PQ \quad (1)$$

$$Q^- + I \rightleftarrows Q^- \cdot I \quad (2)$$

$$Q \cdot PQ \rightleftarrows Q + PQ \quad (3)$$

$$Q + I \rightleftarrows Q \cdot I \quad (4)$$

According to this model, DCMU will bind only to samples at which the binding site is vacant; i.e., with the states Q or Q^-. Following a single preilluminating flash, the equilibrium of eq. (1) is known to be strongly on the side of $Q \cdot PQ^-$ [5,17]; i.e., only a small amount of Q^- is free to react with the inhibitor. The inhibitor binding kinetics may be interpreted to reflect the gradual transformation of $Q \cdot PQ^-$ via $Q^- \cdot PQ$ and Q^- into $Q^- \cdot I$. The equilibria in eq. (1) and (2) are such that eventually almost all centers are in the state $Q^- \cdot I$, provided a sufficient amount of inhibitor is available. This may be concluded from the fact that under equilibrium conditions dark adapted and preilluminated chloroplasts bind almost the same amount of DCMU (fig. 2). The rapid kinetics of DCMU binding in dark-adapted thylakoids ([10], fig. 2) suggest that the equilibrium constant of reaction (4) is substantially larger than that of reaction (3).

As already pointed out, the binding kinetics are pH-dependent [11]. Only at low pH the binding is sufficiently slow to detect substantial kinetics differences in dependence of the preillumination state, with the available sampling technique. It is not yet clear, how the pH effects the binding kinetics. Possibly, the protonation of $Q \cdot PQ^-$ will displace the equilibrium of eq. (1) still further to the left, leaving an even smaller concentration of Q^-. This could explain the slowing down of the binding kinetics for the odd state.

3.2. *Fluorescence experiments*

It is of interest to compare the [^{14}C]DCMU binding kinetics with the rise kinetics of chlorophyll fluorescence upon mixing with DCMU, following a single turnover flash. As inhibitor binding induces transformation of a weakly fluorescent state $Q \cdot PQ^-$ into a strongly fluorescent state $Q^- \cdot I$, it has been assumed that the fluorescence rise is a reliable indicator for the inhibitor binding kinetics. However, fig. 3 and the fluorescence data [11] suggest that this may be only partly true. It is apparent that under comparable conditions there is a substantial amount of [^{14}C]DCMU

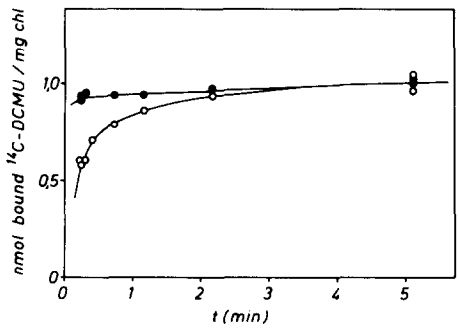

Fig. 2. Kinetics of [^{14}C]DCMU binding at pH 6 with dark-adapted samples (●—●) and with samples preilluminated with one light flash (○—○), total [^{14}C]DCMU conc., 10^{-7} M; chl., 50 μg/ml.

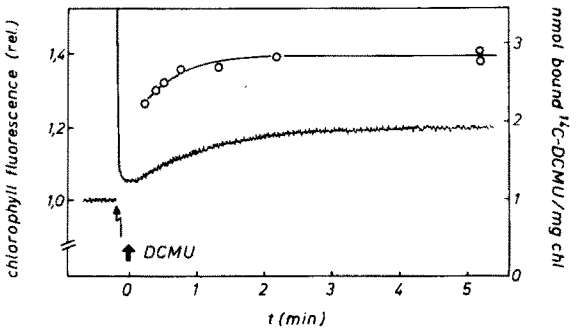

Fig. 3. Comparison of the kinetics of [^{14}C]DCMU binding and of the DCMU-induced rise in chlorophyll fluorescence with samples preilluminated with one saturating light flash. The arrows indicate application of one saturating, single turnover flash and 10 s thereafter injection of DCMU to a final total conc. 3×10^{-7} M. The same experiment carried out with only 10^{-7} M DCMU gave almost no fluorescence rise; chl. was 50 μg/ml.

binding which is not accompanied by a corresponding fluorescence rise. While during the first 15 s fluorescence remains practically unchanged, at this moment already about 70% of [^{14}C]DCMU binding is completed. In this context it is important to note that up to 3 nmol/mg chl, the binding of DCMU was found to be strictly specific and that other PS II inhibitors can quantitatively displace this amount of DCMU [2,3]. It is unlikely that (e.g., under the conditions of fig. 3) 70% of the centers are in the even state despite extended dark adaption and application of a single turnover flash in presence of NH_2OH at pH 6. Hence, one should assume that a substantial amount of DCMU is specifically bound to sites at which binding is not controlled by the redox state of the acceptor side. This assumption is confirmed by the finding that much more DCMU is required to produce a half-maximal dark fluorescence rise (at 10^{-6} M) than to give 50% binding (at 2×10^{-7} M) when centers are in the odd state. With centers in the even state 50% binding was found at 10^{-7} M DCMU. In all these experiments the chlorophyll concentration was identical.

These findings have to be reconciled with fluorescence data [11]. While there is full agreement as far as the properties of the DCMU-induced dark-fluorescence rise is concerned, there may be discrepancies concerning the actual DCMU-binding. It appears that a large amount of DCMU-binding (possibly as much as 75%) is not readily reflected in chlorophyll fluorescence changes, neither in the dark fluorescence rise nor in the fluorescence rise monitored with one additional flash (fig. 1 and 3 of [11]). If analysis of inhibitor binding is based exclusively on fluorescence data (as in [11]) false conclusions may be drawn, as e.g., relating to a large difference in inhibitor association constants for centers in the even and in the odd state: While the fluorescence data [11] suggest a difference by a factor 20, our direct binding data show in the most a factor 2. It has been porposed [11] that there is a type of PS II acceptors (called non-B) the oxidation of which is blocked by rapid DCMU binding before electrons are reversed from $Q \cdot PQ^-$ to Q^-. Possibly, most of the rapid binding in the experiment of fig. 3 can be interpreted as binding to non-B type centers. Actually, following the criteria introduced in [11] (comparison of the fluorescence increase induced by DCMU in the dark and the fluorescence rise induced by one additional saturating flash) the sample of fig. 3 contained about 75% non-B type acceptors.

In conclusion, the presented [^{14}C]DCMU binding data do in principle support the Velthuys model [8,9] of competitive binding of inhibitors and plastoquinone to common binding sites in the vicinity of the primary PS II acceptor Q. Conditions were found where DCMU binding is partially controlled by the redox state of the acceptor complex. However, comparison of the [^{14}C]DCMU binding kinetics and of corresponding fluorescence changes suggests, that redox state dependent DCMU binding may constitute only a part (possibly 25%) of total specific DCMU binding. This finding leaves open the possibility that also only part of the DCMU-binding may be in competition with plastoquinone for a common binding site at PS II.

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