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MODE OF ACTION OF 3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA

EVIDENCE THAT THE INHIBITOR COMPETES WITH PLASTOQUINONE FOR BINDING TO A COMMON SITE ON THE ACCEPTOR SIDE OF PHOTOSYSTEM II

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The kinetics and concentration dependence of the binding of dichlorophenyldimethylurea (DCMU) to Photosystem II (PS II) were monitored through fluorescence measurements. According to whether the acceptor system is in the 'odd' state ($QB^- \rightleftharpoons Q^- B$) or 'even' state (QB), very different results are obtained. The binding to centers in the even state is rapid ($t_{1/2} \simeq 150$ ms at [DCMU] = 10^{-5} M and [chlorophyll] = 10 μg/ml), with a pH-independent rate. The concentration curve of the bound inhibitor (at equilibrium) corresponds to an association constant of about $3.3 \cdot 10^7$ $M^{-1} \cdot 1$. The binding of the inhibitor to centers in the odd state is slow ($t_{1/2} \simeq 3$ s at pH 7, same DCMU and chlorophyll concentrations as above), and depends on pH. In the pH range 6–8, the lower the pH, the slower the kinetics. The association constant is also diminished by a factor of approx. 20 (at pH 7) compared to the even state centers. It is shown that these effects are in good agreement with predictions from Velthuys' hypothesis (Velthuys, B.R. (1981) FEBS Lett. 126, 277–281) that the mode of action of DCMU is a competition with plastoquinone for the binding to the secondary acceptor site. A large part of PS II photochemical quenching corresponds to acceptors which seem to possess a secondary acceptor distinct from B. They were called 'non-B-type acceptors' (Lavergne, J. (1982) Photobiochem. Photobiophys. 3, 257–285) and may be identified with Joliot's 'Q_2' (Joliot P. and Joliot, A. (1977) Biochim. Biophys. Acta 462, 559–574). However, the rate at which the inhibition affects these non-B-type acceptors is similar to the rate of DCMU binding on the B site (i.e., slow in the odd state, fast in the even state).

Introduction

The secondary acceptor B [1] (or R [2]) of PS II is able to store one electron in the vicinity of the primary acceptor Q. This carrier, which is known to be a plastoquinone, is readily reoxidized by the plastoquinone pool when it is doubly reduced (B^{2-}), but not when singly reduced (semiquinone form B^-). Due to this property, it was first assumed that B was a 'special plastoquinone', permanently bound to PS II. However, Goldfeld et al. [3] suggested that B might be an ordinary plastoquinone of the pool, freely mobile in the membrane, except in its semiquinone form which for thermodynamic reasons would have to remain

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PQ, plastoquinone; ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

located at the membrane surface, close to its site of formation. A similar hypothesis has been developped by Rich [4] and by Velthuys [5,6] who suggested that the plastosemiquinone resulting from Q^- reoxidation by a plastoquinone of the pool was tightly bound to a protein in PS II, whereas the affinity of this site for the oxidized (PQ) or doubly reduced (PQH_2) forms of plastoquinone would be much lower. Clearly, this hypothesis on the B mechanism is connected to the view of electron/proton transport across the membrane as a plastoquinone diffusion rather than as a 'bucket brigade' type of transfer [7] through an immobilized plastoquinone chain.

Velthuys further suggested [5,6] that the mode of action of inhibitors of the DCMU type (which block Q^- reoxidation by B) could be a competition with plastoquinone for the occupancy of the B^- binding site. This hypothesis offers the advantage of providing a simple explanation for a phenomenon which otherwise requires an ad hoc assumption, i.e., the back-transfer of an electron from B^- to Q occurring in the dark upon DCMU addition [2]. The acceptor system may be found either storing one electron, as Q^- or QB^- (we denote these two forms the 'odd state' of PS II) or none, as in the 'even state' Q, equivalent to state 'Q-B' in the classical formulation. Considering the equilibrium occurring in the odd state $QB^- \rightleftharpoons Q^- + PQ$ (with an equilibrium constant of about 20 in favor of QB^-, according to Diner [8]), and assuming that the inhibitor binds to Q or Q^-, one predicts that DCMU will bind at a 20-fold slower rate to centers in the odd state compared to the even state. This binding corresponds to the reaction $Q^- + I \leftrightarrow Q^-I$ (where I represents the inhibitor), and thus, at saturating DCMU concentration, a total displacement within the odd state centers towards form Q^-I is expected.

The Goldfeld-Rich-Velthuys hypothesis of a tight binding of only the semiquinone form of plastoquinone to PS II has found support from studies using exogenous quinones, such as DBMIB [6] or benzoquinone [9]. In this paper we investigate the binding properties of DCMU to PS II centers in the even and odd states, providing more direct evidence that only the B^- form is tightly associated with the center and that DCMU competes with plastoquinone for the occupancy of a unique site.

In previous papers [10–13] we have reported evidence for a heterogeneity of PS II primary acceptors, showing that in addition to the already mentioned 'B-type' acceptors, one had to consider 'non-B-type' Qs. Beyond the fact that they are not involved in the $QB^- \xrightarrow{DCMU} Q^-B$ reaction, these non-B-type acceptors possess several other distinctive characteristics. The present investigation on DCMU binding will provide some new information on this problem.

Material and Methods

Broken Spinach chloroplasts were isolated as previously described [10]. They were used at a concentration of about 10 μg chlorophyll/ml in a sucrose (0.4 M), $MgCl_2$ (5 mM) medium, buffered with 50 mM Hepes (pH 7 or 8) or Mes (pH 6). The suspension was dark adapted for 5 min with 50 μM ferricyanide in order to fully oxidize B^-, then incubated for 10 min with 1 mM hydroxylamine (or CCCP, where indicated) before running the experiments.

The experimental set up has been described in Refs. [10 and 12]. A chloroplast sample may be preilluminated by one or several (300 ms apart) saturating flashes (F_1) of a few microseconds duration. 1 s later, it is rapidly mixed with suspension medium containing DCMU. A variable time after the mixing the fluorescence excited by a weak light pulse is measured. The sample may be further illuminated by a saturating flash F_2 (1 μs duration) from a dye laser. An analytical pulse is then triggered in order to record the fluorescence yield 100 ms after F_2.

Results

Principle of the measurement

The fluorescence yield (Φ) of chlorophyll is used as an indicator of the redox state of the primary acceptors: dark-adapted chloroplasts with 100% Q oxidized have a basal fluorescence yield Φ_0, whereas the total reduction of the Qs corresponds to a maximum yield Φ_{max} (approx. $3\Phi_0$). In the absence of inhibitor, Φ decays rapidly in the dark after flash illumination, due to the rapid reoxidation of Q^- by the secondary acceptor

(milliseconds range). With DCMU present, however, this reaction is blocked, and the Φ decay is slowed down to the seconds time range (or still slower in the presence of an exogenous donor such as hydroxylamine or CCCP which preclude the charge recombination of Q^- with the oxidant stored on the donor side). Accordingly, one can estimate the amount of DCMU-blocked acceptors by measuring the fluorescence increase ($\Delta\Phi$) above Φ_0 subsisting a hundred milliseconds after a saturating test flash. Before mixing with DCMU, the PS II centers may have been prepared in the odd state (QB^-) by one preillumination flash following dark adaptation in the presence of an oxidant (50 μM ferricyanide). Denoting the test flash F_2, the binding of DCMU is estimated from $\Delta\Phi(0F_1, 1F_2)$ for the even state, and from $\Delta\Phi(1F_1, 1F_2)$ for the odd state. However, in the latter case, the dark DCMU-induced reaction, $QB^- \overset{DCMU}{\rightarrow} Q^- B$, provides further information on DCMU binding. The corresponding fluorescence rise will be denoted $\Delta\Phi(1F_1, 0F_2)$. In chloroplasts where normal oxidizing equivalent storage occurs on the oxygen-evolving system, the Q^- formed by the dark DCMU-induced reaction is progressively reoxidized through charge recombination. Odd state centers are thus unstable upon DCMU addition. In order to stabilize them, we used hydroxylamine [14] or CCCP [15] which block the recombination reaction by rapidly rereducing the PS II donor side.

If things were simple, $\Delta\Phi(1F_1, 0F_2)$ should reflect all the DCMU-inhibited acceptors in the odd state, and no further increase in $\Delta\Phi$ should be caused by F_2. However, this is not so, F_2 does reveal DCMU-inhibited Qs which did not show up through the DCMU-induced back-transfer. Actually, this, and more generally the fact that $\Delta\Phi(1F_1, 0F_2)$ accounts only for 25–50% of $\Delta\Phi_{max}$, was at the origin of the distinction between B-type and non-B-type acceptors [10–13]. In this conceptual framework, the difference $[\Delta\Phi(1F_1, 1F_2) - \Delta\Phi(1F_1, 0F_2)]$ (which we shall denote in a shorter way $\Delta\Phi(1F_1 \pm F_2)$) indicates the amount of DCMU-inhibited acceptors of the non-B type.

Kinetics of DCMU binding

Fig. 1 shows typical time courses of $\Delta\Phi(0F_1, 1F_2)$, $\Delta\Phi(1F_1, 0F_2)$ and $\Delta\Phi(1F_1, 1F_2)$, following rapid mixing with a saturating concentration of DCMU, at pH 7 or 8. The chloroplasts were first incubated with 50 μM ferricyanide (in order to set all centers in the even state), then with 1 mM hydroxylamine. The points plotted at time zero were measured before introducing DCMU into the mixing medium. It can be seen that in the absence of inhibitor, the fluorescence yield has not totally relaxed to Φ_0 100 ms after F_2, particularly at low pH (this was already noticed by Diner and Joliot [16]). We do not know whether this very slow phase indicates a slow reoxidation of part of the Q^- or is due to another phenomenon, and this problem will be ignored in the following studies. A salient feature of the data shown in Fig. 1 is that the rate of DCMU binding is markedly slowed down in the one-flash preilluminated samples, particularly at low pH (see also Table I). It was checked that this phenomenon was controlled by the B/B^- binary mechanism: the kinetics obtained after two preilluminating flashes are close to that of $\Delta\Phi(0F_1, 1F_2)$, whereas after three F_1, they resemble the one-F_1 time courses.

Although its amplitude is about 2-times larger, the kinetics of $\Delta\Phi(1F_1, 1F_2)$ are very similar to those of $\Delta\Phi(1F_1, 0F_2)$. However, a plot of the difference $\Delta\Phi(1F_1 \pm F_2)$ sometimes reveals a minor faster contribution, with a rate similar to that of the $\Delta\Phi(0F_1, 1F_2)$ kinetics. This may be attributed to the presence of centers in the even state due to 'misses' in the even-to-odd transition elicited by F_1 (and perhaps to a nontotal oxidation of B^- during the dark adaptation).

Whereas the rate of DCMU fixation on centers in the even state ($\Delta\Phi(0F_1, 1F_2)$) does not vary significantly with pH, a marked pH dependence was found for the odd state. This appears in Fig. 1, and also in Table I which shows that the kinetics are still slower at pH 6.

CCCP [15] is a member of the 'ADRY' [17] family: it promotes a rapid rereduction of the positive charge stored on the PS II donor side. It thus blocks the recombination reaction of Q^- as efficiently as hydroxylamine. However, in contrast to the latter, it does not inactivate the oxygen-evolving system. Fig. 2 shows the kinetics of DCMU binding in the presence of CCP. The observed behavior is qualitatively the same as in

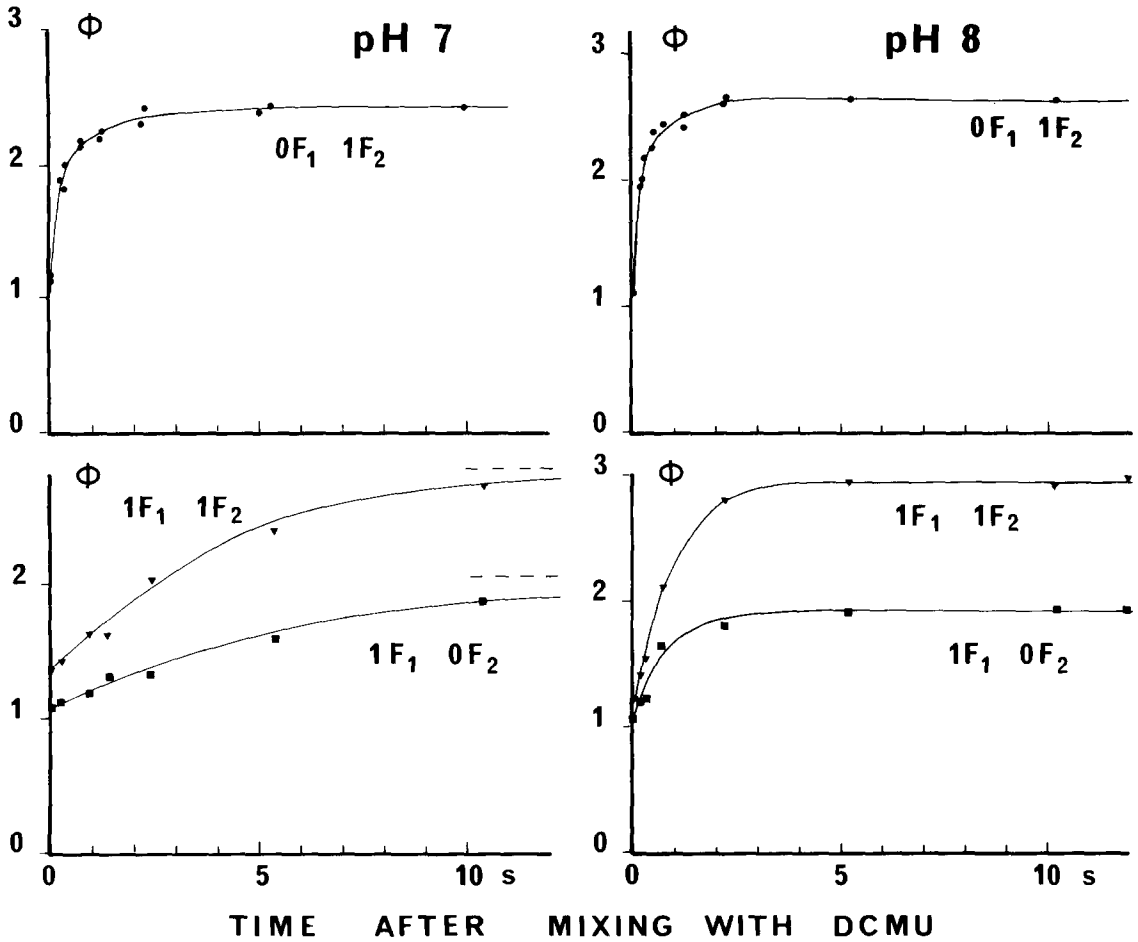


Fig. 1. Kinetics of $\Delta\Phi(0F_1, 1F_2)$ (above), $\Delta\Phi(1F_1, 0F_2)$ and $\Delta\Phi(1F_1, 1F_2)$ (bottom) at pH 7 (left) or 8 (right). The time scale origin is the beginning of the mixing with DCMU (final concentration 10^{-5} M). The vertical scale gives the fluorescence yield Φ in units of Φ_0 (dark-adapted level). The dashed lines in the bottom left figure indicate the final levels reached by the two curves (at 20 s). The chloroplast suspension was first incubated with 50 μM ferricyanide for 5 min, then with 1 mM hydroxylamine for 10 min.

TABLE I

$t_{1/2}$ OF DCMU BINDING TO PS II CENTERS IN THE EVEN OR ODD STATE

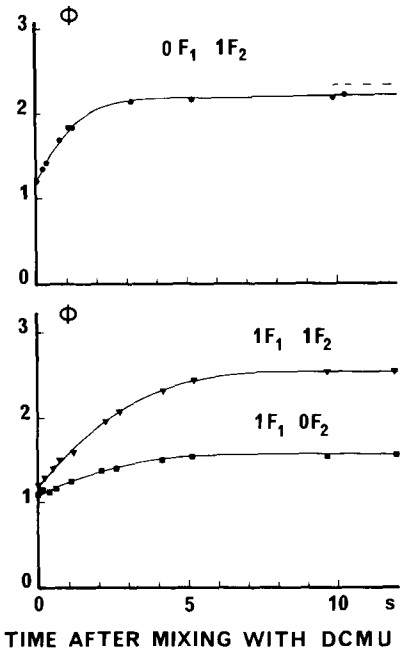
The half-times indicated are those of $\Delta\Phi(0F_1, 1F_2)$ and $\Delta\Phi(1F_1, 0F_2)$ measured from the same batch of chloroplasts, (also used in the experiment of Fig. 2), with 10^{-5} M DCMU and 10 μg/ml chlorophyll.

	$0F_1$ $1F_2$	$1F_1$ $0F_2$
pH 6 (NH_2OH)	150 ms	7.0 s
pH 7 (NH_2OH)	150 ms	2.5 s
pH 7 (CCCP)	950 ms	3.4 s
pH 8 (NH_2OH)	150 ms	550 ms

the presence of hydroxylamine: fast kinetics for $\Delta\Phi(0F_1, 1F_2)$ and slow kinetics for both $\Delta\Phi(1F_1, 0F_2)$ and $\Delta\Phi(1F_1, 1F_2)$ with only a small faster contribution to the latter. However, compared to the hydroxylamine experiment, a marked slowing down of the three curves is observed (see Table I). Another feature observed in the presence of CCCP is the occurrence of a small slow component in the $\Delta\Phi(0F_1, 1F_2)$ kinetics.

Concentration curves

We are now interested in the equilibrium levels of $\Delta\Phi$ as a function of the DCMU concentration.



They are measured 5 min after DCMU addition to ensure complete equilibration. Fig. 3 shows the concentration curves obtained at pH 7, in the presence of hydroxylamine. The $\Delta\Phi(1F_1, 0F_2)$ level is still hardly affected at 10^{-7} M DCMU, whereas $\Delta\Phi(0F_1, 1F_2)$ has reached about 75% of its saturation level. The half-saturating concentrations are, respectively, $3 \cdot 10^{-8}$ and $6.5 \cdot 10^{-7}$ M for $\Delta\Phi(0F_1, 1F_1)$ and $\Delta\Phi(1F_1, 0F_2)$.

The concentration dependence of $\Delta\Phi(1F_1, 1F_2)$ is roughly similar to that of $\Delta\Phi(1F_1, 0F_2)$. However, if one substracts the latter contribution, the concentration curve of the difference $\Delta\Phi(1F_1 \pm 1F_2)$ is significantly different. It varies within a

Fig. 2. Same as Fig. 1, except hydroxylamine was replaced by 10^{-5} M CCCP. Chloroplast suspension at pH 7. The half-times obtained on the same batch of chloroplasts under various conditions are indicated in Table I. The dashed line indicates the final level of curve $\Delta\Phi(0F_1, 1F_2)$ reached at 25 s.

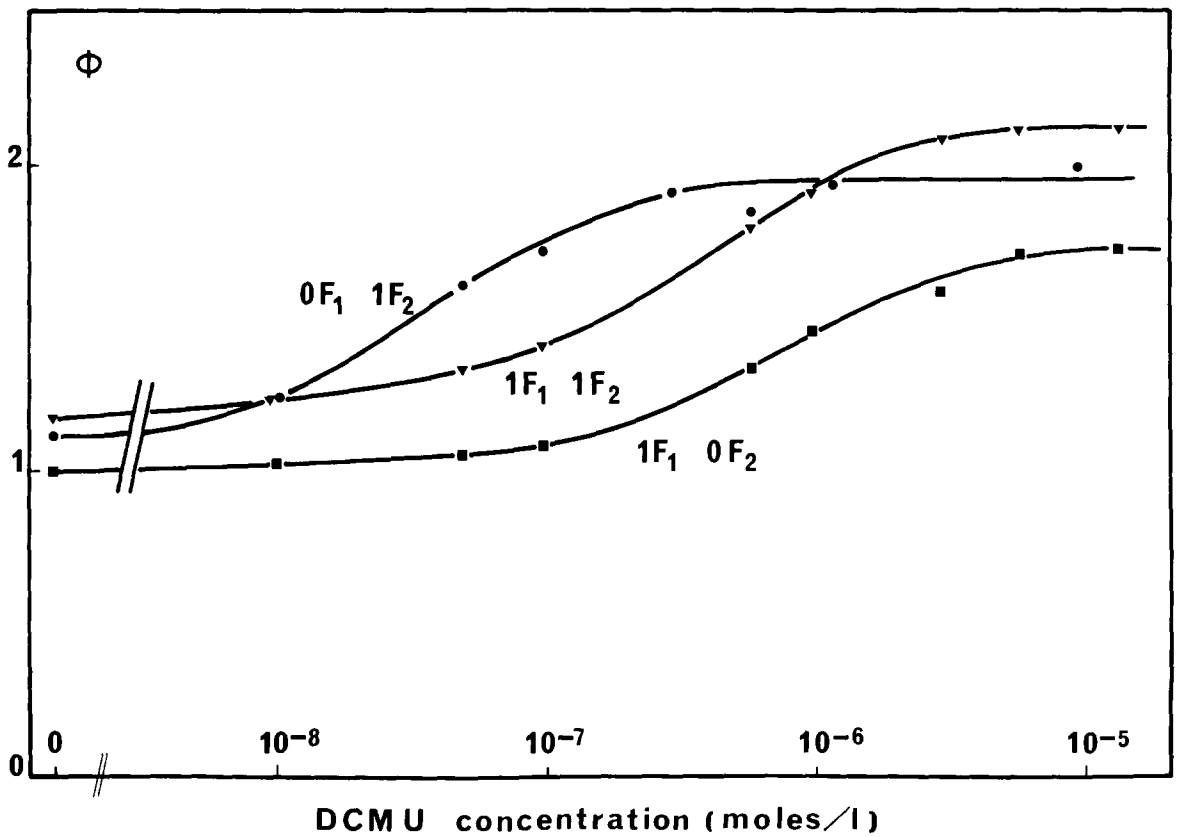


Fig. 3. Concentration dependence of $\Delta\Phi(0F_1, 1F_2)$, $\Delta\Phi(1F_1, 0F_2)$ and $\Delta\Phi(1F_1, 1F_2)$. Each point was measured 5 min after mixing with DCMU at the indicated (final) concentration. Chloroplasts suspension at pH 7, in the presence of 1 mM hydroxylamine.

wide concentration range, and starts rising at lower concentrations than $\Delta\Phi(1F_1, 0F_2)$.

Discussion

Binding site of DCMU

The results reported above show that DCMU binds markedly slower and with a lower association constant to PS II centers in the odd state. This finding is in full agreement with the hypothesis proposed by Velthuys [5,6], namely: (i) B is an ordinary plastoquinone of the pool, freely diffusing in the membrane in its oxidized (PQ) or fully reduced (PQH_2) forms. Only the semiquinone form (PQ^-) is tightly bound to PS II (odd state). And, (ii) DCMU (and similar inhibitors) binds at the same site as B^-. In terms of enzymology, DCMU may be called a competitive inhibitor of the 'Q-plastoquinone oxidoreductase'.

Denoting I as the inhibitor, one has the reactions:

$$\left.\begin{array}{l} Q + I \underset{\beta}{\overset{\alpha}{\leftrightarrow}} QI \\ Q^- + I \underset{\beta}{\overset{\alpha}{\leftrightarrow}} Q^-I \end{array}\right\} \quad (1)$$

$$QB^- \leftrightarrow Q^- + PQ \quad (2)$$

The association and dissociation rate constants α and β are assumed to be unaffected by the state of Q. The association (equilibrium) constant of I is thus $K_I = \alpha/\beta$. The constant corresponding to equilibrium 2, i.e., $K = [QB^-]/[Q^-][PQ]$, is not identical to that estimated by Diner [8], $K_2 = [QB^-]/[Q^-B]$, in the framework of the classical QB model where only first-order reactions take place. Diner obtained $K_2 = 15$ by fitting experimental equilibrium curves $[Q^-] = f([PQ^{2-}])$ obtained in a situation where almost all centers are in the odd state. The theoretical equation was:

$$\frac{[Q^-]}{[Q_{tot}]} = 1 - \left(1 + \frac{1}{K_2} + \frac{[PQ^{2-}]}{K_1 K_2}[PQ]\right)^{-1} \quad (3)$$

Where Diner took: $[Q]_{tot} = 1$, $[PQ]_{tot} = 20$, $K_1 = 1$ (K_1 is the equilibrium constant $[B]\,[PQ^{2-}]/[B^{2-}][PQ]$, its value was determined in Ref. 18).

In the model described by Eqns. 1 and 2, the equivalent relation is:

$$\frac{[Q^-]}{[Q_{tot}]} = (1 + K[PQ])^{-1} \quad (4)$$

No analytical identification can be made between Eqns. 3 and 4. However, a very close fit is obtained between Eqn. 3 using $K_2 = 15$, and Eqn. 4 using $K = 0.8$ (and identical values for the other parameters). It thus turns out that the satisfactory fitting obtained by Diner is not an argument in favor of the classical QB view against the free plastoquinone model. The latter is consistent with Diner's data, taking $K = 0.8$. Denoting $K' = K[PQ_{tot}]$ the equilibrium ratio $[QB^-]/[Q^-]$ when the pool is totally oxidized (as in our experiments), one finds $K' = 16$, close to Diner's value for $K_2 = [QB^-/[Q^-P]$. Although the two models imply different mechanisms, they both fit Diner's data, taking $K' \simeq K_2$. Several corrections were envisaged by this author who finally proposed $K_2 = 15$–20. We can thus assign the same range of values to K'.

Coming back to the problem of DCMU binding, it is clear that if equilibrium 2 is rapid, then at every instant a fraction $1/(1 + K')$ of the odd state centers is in state Q^-, the remaining (fraction $K'/(1 + K')$) being in the state QB^- unfit for DCMU binding. The rate of inhibitor fixation is therefore decreased by a factor $1/(1 + K')$ compared to the even state. Consequently, the apparent association constant for DCMU is decreased by the same factor.

At pH 7, the results reported in Table I and Fig. 3 give a ratio of 17 for the rates and 22 for $I_{1/2}$. The average value obtained in several experiments at this pH is about 20 for both quantities, i.e., close to the expected value of $(K' + 1)$. This consistency provides serious support to the Velthuys model, and to the various simplifying assumptions we have made (i.e., neglecting the association constants of PQ and PQH_2 to the center, neglecting the possible influence of the state of Q on α and β).

The pH affects the rate of DCMU binding on odd state, but not on even state centers. This suggests that K is pH dependent, increasing when the pH is decreased. However, this dependence is not great enough to be accounted for by a protonation of B^- ($Q^- + PQ + H^+ \rightleftharpoons QBH$). More

likely, the equilibrium may be influenced by surface charges on the membrane, which in turn depend on pH. From the concentration curve of Fig. 3, the association constant of DCMU to PS II centers (even state) is $K_1 = (I_{1/2})^{-1} = (3 \cdot 10^{-8})^{-1}$ $1 \cdot M^{-1}$. Since the half-time for the inhibitor binding is $t^b_{1/2} \simeq 0.15$ s (at 10^{-5} M DCMU), the half-time for DCMU release may be computed from:

$$t^r_{1/2} = t^b_{1/2} \cdot 10^{-5} \cdot K_1$$

One obtains $t^r_{1/2} = 50$ s which is comparable to the 80 s value found by Bouges-Bocquet et al. [19]. It is interesting to note that the very efficient blocking of Q^- in the presence of DCMU and hydroxylamine (Q^- reoxidation occurs in the half-hour time range) is partially due to the B/B^- mechanism: in its absence, the electrons would leak from Q^- with a time constant of the order of $t^r_{1/2}$, since the rebinding time of the inhibitor is much slower than Q^- normal reoxidation. What happens is that the electrons do leak, but are readily back-transferred from B^- to Q upon DCMU rebinding.

The effect of CCCP on the rates of DCMU binding confirms Homann's [20] finding that this substance does not interact only with the PS II donor side, but also with the acceptor side. The simpler way to account for the slowing down of DCMU binding is to assume that CCCP also may bind to PS II secondary acceptor site. CCCP would be an inhibitor of the DCMU type, although with a low association constant. Let F be the equilibrium ratio [QC]/[Q] (or $[Q^-C]/[Q^-]$) at a given CCCP (denoted by C) concentration. The proportion of centers with a vacant binding site is $(1 + F)^{-1}$ among even state centers, and $(1 + K' + F)^{-1}$ among odd state centers. Thus, one expects a greater effect of CCCP on the rate of DCMU binding to even state centers. This was indeed the case in the experiment of Fig. 2. From the effect on $\Delta\Phi(0F_1, 1F_2)$, i.e., a 6-fold slower rate in the presence of 10^{-5} M CCCP, one obtains $F = 5$. In the odd state, one then expects the CCCP effect to be like the ratio $(1 + K' + F)/(1 + K')$, i.e., 1.3 (taking $K' = 16$ from Table I). This fits satisfactorily with the experimental value of 1.4. In order to explain that the CCCP inhibition (of 5/6 of the centers) is not revealed by the $\Delta\Phi(0F_1, 1F_2)$ measurement in the absence of DCMU, one must assume that the turnover of CCCP is fast so that the relaxation of the odd state towards equilibrium is achieved in the 100 ms time interval after F_2. If this interpretation of the CCCP effect is correct, one expects high concentrations of CCCP to cause a back transfer $QB^- \rightarrow Q^-$ similarly to DCMU. We checked that it was indeed the case, obtaining period 2 oscillations of $\Delta\Phi(nF_1, 0F_2)$ (chloroplasts incubated with 1 mM hydroxylamine) in an experiment involving mixing with CCCP ($5 \cdot 10^{-5}$ M) instead of DCMU. However, the amplitude was only about one-fourth that obtained with DCMU, whereas the expected value would be about one-half, a point for which we have no simple explanation. The hypothesis of a competition between DCMU and CCCP is consistent with the fact that both compounds have definite structural similarities, as already noticed by Etienne [21]. In particular, CCCP possesses the NH side group common to DCMU and similar inhibitors, although not in form $-\overset{\|}{C}-NH$ considered to be the basic structural element essential for inhibition (see Ref. 22).

Heterogeneity of the acceptors

As already mentioned, according to Refs. 10–13, we believe that the quenching corresponding to the large gap between Φ_{max} and $\Delta\Phi(1F_1, 0F_2)$ (at its full extent) is mostly due to distinct primary acceptors called non-B type. Several experiments suggested that B-type acceptors (abbreviated Q(B)) and non-B-type acceptors (abbreviated $Q(\bar{B})$) were both present in the same PS II centers, operating in parallel with a priority rule in favor of Q(B). A first photoact causes the reduction of Q(B), a second one that of $Q(\bar{B})$. Both photoacts may take place during a single flash (double hitting) if the duration of the flash is long compared to the rereduction of the center chlorophyll P-680.

A similar model of centers with two parallel acceptors Q_1 and Q_2 has been first proposed by Joliot and Joliot [23,24]. One operational definition of Q_1 is that one saturating flash in the presence of DCMU and hydroxylamine at pH 6.5 causes the reduction of (about) all Q_1 and no Q_2. Many features (detailed in Ref. 13) suggest an

identification of (respectively) the B- and non-B-type acceptors to Q_1 and Q_2. However, in Ref. 13 we reported a large gap between $\Delta\Phi(1F_1, 0F_2)$ and $\Delta\Phi(0F_1, 1F_2)$ (in the presence of hydroxylamine, pH 6), which was interpreted by stating that the B-type acceptors were a subset of the Q_1s. Actually, we now believe (see, e.g., Fig. 1, left; in the present paper) that this gap was overestimated in these earlier experiments, due to an insufficient equilibration time after DCMU mixing in the $\Delta\Phi(1F_1, 0F_2)$ measurement. Accordingly, the identity Q_1-B-type acceptor, Q_2-non-B type may be assumed in the following.

The analysis developed in the first part of this section was restricted to the Q(B) inhibition by DCMU, We now wish to consider the information we have obtained about the inhibition of $Q(\bar{B})$. $\Delta\Phi(1F_1, 0F_2)$ is purely a Q(B) indicator, and the difference $\Delta\Phi(1F_1 \pm F_2)$ is purely a $Q(\bar{B})$ indicator (neglecting the amount of even state centers after 1F, due to odd state centers still present after dark adaptation with ferricyanide, and to misses in the F_1-induced transition). What about $\Delta\Phi(0F_1, 1F_2)$? In the absence of hydroxylamine or Tris treatment, one saturating microsecond flash elicits the reduction of both Q(B) and $Q(\bar{B})$ in two successive acts, due to the submicrosecond rereduction of the center chlorophyll. The treatments mentioned above inactivate the oxygen-evolving system and slow the rereduction rate of P^+-680 down to the microsecond range, especially at low pH [25]. Thus, only one photoact per flash can occur in the presence of hydroxylamine at low pH (6–6.5), whereas at higher pH more double hitting seems to occur. Accordingly, $\Delta\Phi(0F_1, 1F_2)$ should be a purely Q(B) indicator under single-turnover conditions, but be contaminated by $Q(\bar{B})$ when double hitting is important (in the absence of hydroxylamine, as in the CCCP experiment of Fig. 2, or hydroxylamine at pH 7–8). We have thus experimental information on DCMU binding to $Q(\bar{B})$ both when centers are in the odd state (through the difference $\Delta\Phi(1F_1 \pm F_2)$), and when they are in the even state where $Q(\bar{B})$ contributes to $\Delta\Phi(0F_1, 1F_2)$ except in the presence of hydroxylamine at low pH.

Now, it turns out that no independent kinetic contribution of $Q(\bar{B})$ seems to show up in the DCMU-binding kinetics of Figs. 2 and 3. The rate of $Q(\bar{B})$ inhibition seems to be identical to that of Q(B), both in the even and odd states of the center, i.e., controlled by the B^- binding. It may be envisioned that one single site controls two independent acceptors. This is, however, unlikely, since no redox equilibration between Q(B) and $Q(\bar{B})$ should occur (otherwise the binary phenomena caused by B/B^- would be scrambled), and since indications exist that $Q(\bar{B})$ may be located close to the membrane surface opposite to Q(B) [12,24]. A more satisfactory hypothesis could be that the redox state of $Q(\bar{B})$ does not influence the fluorescence yield as long as Q(B) is oxidized. This is somewhat expected from the priority of Q(B) photoreduction which suggests that the rate of electron transfer from P*-680 to Q(B) is much faster than to $Q(\bar{B})$. The excitation would not 'sense' the state of $Q(\bar{B})$ unless Q(B) is reduced. Hence, states $Q(B)Q(\bar{B})$ and $Q(B)Q(\bar{B})^-$ would be equally quenching states (level Φ_0), $Q(B)^- Q(\bar{B})^-$ a low-quenching state (Φ_{max}), and $Q(B)^- Q(\bar{B})$ an intermediate state. With this hypothesis, our kinetic results are accounted for if one assumes that the binding of DCMU to $Q(\bar{B})$ is fast, i.e., with a rate similar to or faster than that of Q(B) in the even state. In the odd state, the binding to Q(B) would be a limiting process to reveal the amount of blocked $Q(\bar{B})$.

The concentration dependence (Fig. 3) of the difference $\Delta\Phi(1F_1 \pm 1F_2)$ should reflect the same phenomenon and behave like $\Delta\Phi(1F_1, 0F_2)$. However, this is not quite true, since $\Delta\Phi(1F_1 \pm 1F_2)$ starts rising at lower concentrations than $\Delta\Phi(1F_1, 0F_2)$. This may indicate a higher affinity for DCMU of the $Q(\bar{B})$ site than of the Q(B) site (odd state). Alternatively, one cannot rule out that this curve may be distorted by a contribution from even state centers.

Further work is obviously needed to ascertain the existence of an independent binding site for DCMU on $Q(\bar{B})$ and describe its characteristics. At any rate, a point which receives substantial support from the data we have reported is the notion that $Q(\bar{B})$ belongs to the same photochemical center as Q(B).

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