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COMPETITION BETWEEN PLASTOQUINONE AND 3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA AT THE ACCEPTOR SIDE OF PHOTOSYSTEM II

WALTER OETTMEIER and HANS-JOACHIM SOLL

Lehrstuhl Biochemie der Pflanzen, Ruhr-Universität, D-4630 Bochum 1 (F.R.G.)

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In plastoquinone-depleted thylakoids, as obtained by *n*-hexane extraction, a high specifically labeled 3-(3,4-[^{3}H]dichlorophenyl)-1,1-dimethylurea ([^{3}H]DCMU) is displaced competitively from the membrane by the short-chain plastoquinone analogue plastoquinone-1. A binding constant $K_b = 51 \pm 19$ μM could be calculated for plastoquinone-1. Similarly, [^{3}H]DCMU is also competitively displaced by the Photosystem II acceptor dichlorophenolindophenol ($K_b = 20 \pm 3$ μM).

DCMU in photosynthesis research is the most commonly used inhibitor. Simultaneously, it represents a class of highly efficient herbicides. Most recently, a 32–34 kDa protein within the thylakoid membrane has been found to be the binding protein for this type of inhibitor [1,2]. Similarly, 41–55 kDa proteins – related to the PS II reaction center – were found to be the binding sites for phenolic inhibitors, which like DCMU block electron transport at the reducing side of PS II [2,3]. Although the binding sites of both types of inhibitors are now known, their mechanism of action, i.e., how inhibition of photosynthetic electron transport is brought about, remains unclear. From flash-induced absorbance changes of plastosemiquinone in the presence of different inhibitors, Velthuys [4] has inferred an electron-dependent competition between inhibitor and plastoquinone. This was further substantiated in fluorescence experiments by Lavergne [5]. Direct evidence for a competition between inhibitor and plastoquinone, however, has not yet been obtained. We wish to report here that PQ-1 in plastoquinone-depleted thylakoids competitively displaces a high specifically labeled [^{3}H]DCMU from the membrane, although affinities of DCMU and PQ-1 for the binding site differ by a factor of more than a thousand. A similar competition was also observed between [^{3}H]DCMU and the PS II acceptor DCIP.

[^{3}H]DCMU, originally synthesized at a specific activity of 3.54 Ci/mmol in 1974, was a generous gift from Dr. I. Ohad, Department of Biochemistry, The Hebrew University of Jerusalem, Israel. For purification from autoradiolysis products it was rechromatographed on prewashed (three times), precoated silicagel plastic sheets (Kieselgel 60 F 254, Merck AG, Darmstadt, F.R.G.) with benzene/acetone (2 : 1, v/v) as the solvent. The zone corresponding to DCMU ($R_f = 0.71$) was cut out and eluted with methanol. The concentration of DCMU was determined from its absorption at 251 nm ($\epsilon = 23\,500\ M^{-1} \cdot cm^{-1}$) in methanol. PQ-1 was synthesized according to the method of Scott [6].

Chloroplasts from spinach were prepared following a procedure by Nelson et al. [7]. Hexane extraction of lyophilized chloroplasts for removal of endogenous plastoquinone was performed

Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PQ-1, plastoquinone-1; PS, photosystem; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

according to the method of Hirayama and Kabata [8] using 5 ml *n*-hexane per mg Chl. All operations were carried out in a glove box in an atmosphere of dry nitrogen. Reconstitution of hexane-extracted chloroplasts was achieved by adding back a 10-fold excess (on the original Chl basis) of hexane extract and evaporation of the solvent in the vacuum.

Photosynthetic activities of chloroplast preparations were assayed using DCIP as the electron acceptor. DCIP reduction was followed at 600 nm in a Zeiss PMQII spectrophotometer modified for cross-illumination with actinic light ($0.2\ W \cdot cm^{-2}$). The reaction medium contained, in a volume of 2 ml, 30 mM Hepes buffer, pH 7.0, 3 mM $MgCl_2$, 30 μM DCIP, 7 μg gramicidin and chloroplasts at a concentration corresponding to 3–50 μg Chl. If diphenylcarbazide was used as the electron donor, 1.125 mM diphenylcarbazide was added. The chloroplast preparations had the following activities: control chloroplasts: H_2O/DCIP, 80.5; diphenylcarbazide/DCIP, 92.4; hexane-extracted chloroplasts: H_2O/DCIP, 1.8; diphenylcarbazide/DCIP, 10.2; reconstituted chloroplasts: H_2O/DCIP, 42.9; diphenylcarbazide/DCIP, 53.1 μmol DCIP reduced/h per mg Chl.

Binding and displacement experiments with [^{3}H]DCMU were performed essentially as described recently (Method B [9]). Counts were corrected for quenching by the buffer system and pigments as well.

Binding studies of radioactively labeled inhibitors of photosynthetic electron transport to isolated thylakoids allow determination of the binding constant K_b, which is equal to the inhibition constant K_i extrapolated to zero Chl concentration, the number of binding sites x_t, and the number of Chl molecules per bound inhibitor. The latter is a measure of the number of bound inhibitor molecules per electron-transport chain [10]. Furthermore, binding experiments of a labeled inhibitor in the presence of an unlabeled inhibitor or compound yield information on the type of competition. If competitive binding occurs K_b of the unlabeled compound can be calculated [10].

For elucidation of the mechanism of action of DCMU it was of special interest to evaluate whether DCMU and plastoquinone indeed have binding sites in common. These studies were facilitated by the availability of a high specifically labeled [^{3}H]DCMU (3.54 Ci/mmol). So far, DCMU-binding with spinach chloroplasts have been performed only with [^{14}C]DCMU of low specific activity of 0.99 mCi/mmol [11]. The binding curve of [^{3}H]DCMU to isolated thylakoids in the nanomole range is shown in Fig. 1. Binding curve and binding parameters agree well with those reported (Table I). The high specific activity of [^{3}H]DCMU allowed binding studies also in the picomole range (inset, Fig. 1). In this region predominantly specific binding with little unspecific binding occurs (see Refs. 10 and 11) and the relation betwen free and bound DCMU is absolutely linear.

In the competition experiments the short-chain analogue PQ-1 instead of native plastoquinone has been used, since plastoquinone in sufficiently high concentrations can be kept in solution only in the presence of detergents (see Ref. 12). Excess detergents might affect the binding properties of the inhibitor.

PQ-1 is not very effective in displacing $5 \cdot 10^{-9}$ M DCMU (corresponding to 2.5 pmol free and 7.5 pmol bound DCMU; see inset, Fig. 1) from a thylakoid preparation (Fig. 2). Even at the highest concentration applied ($5 \cdot 10^{-4}$ M), i.e., in a 10^6-fold excess, PQ-1 displaces [^{3}H]DCMU to only 25% from the membrane. This small displacement does not allow for evaluation of the type of competition and may be due to the high endogenous plastoquinone content of the thylakoid membrane.

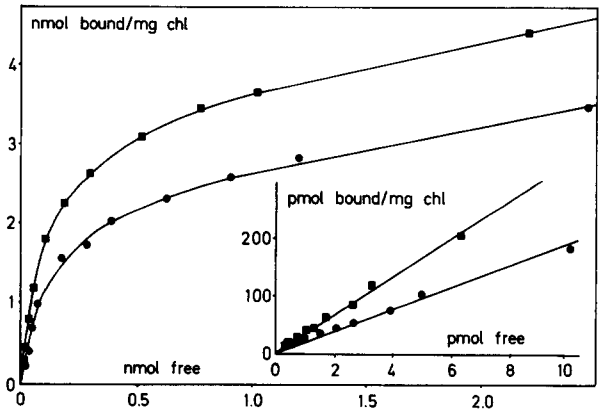

Fig. 1. Binding of [^{3}H]DCMU to control (■———■) and plastoquinone-depleted (●———●) thylakoid membranes. Inset: binding in the picomole range.

TABLE I

BINDING PARAMETERS OF [^{3}H]DCMU IN CONTROL AND PLASTOQUINONE-DEPLETED MEMBRANES

Values in parentheses are taken from Ref. 11.

Concentration (μM)	K_b (μM)	x_t (nmol/mg Chl)	Molecules Chl/DCMU
(A) control			
–	0.034	2.36	472
–	(0.019±0.013)	(3.8±0.9)	(292±91)
(B) Plastoquinone-depleted			
(1) in the presence of PQ-1			
–	0.031	1.60	695
44	0.104	1.47	754
133	0.257	1.69	659
303	0.364	1.61	691
(2) in the presence of DCIP			
–	0.039	1.42	781
5	0.143	1.37	837
15	0.175	1.29	860
50	0.276	1.27	873

Further displacement experiments, therefore, have been performed with thylakoid preparations, which have been depleted of plastoquinone by hexane extraction [8,13]. [^{3}H]DCMU binding in plastoquinone-depleted thylakoids is generally lower as compared to untreated thylakoids (Fig. 1). The binding constant did not change very much, whereas the number of binding sites decreased (Table I). The binding data correspond well to the inhibition data. The following pI_{50} values for DCMU (system diphenylcarbazide/DCIP) were observed: control chloroplasts. 7.04; extracted chloroplasts, 6.89; reconstituted chloroplasts, 6.98. This indicates that the DCMU-binding site is little affected by the extraction procedure.

In plastoquinone-depleted chloroplasts, however, [^{3}H]DCMU was efficiently dislodged by PQ-1, causing loss of more than 80% of the originally bound [^{3}H]DCMU at the highest concentration of PQ-1 applied (Fig. 2). In reconstituted chloroplasts again little displacement of [^{3}H]DCMU by PQ-1 is observed, i.e., reconstituted chloroplasts closely resemble control chloroplasts (Fig. 2). This further corroborates the notion that endogenous plastoquinone in fact interferes with PQ-1 displacement of DCMU.

Double-reciprocal plots for [^{3}H]DCMU binding in the presence of several concentrations of PQ-1 together with the control are depicted in Fig. 3. The corresponding binding parameters are listed in Table I. As is indicative of a common y-intercept of all regression lines (Fig. 3), and different binding constants K_b but almost identical number of binding sites x_t (Table I), there exists a true competitive interaction between DCMU and PQ-1. This notion could be further supported by plotting the binding data according to Eadie-Scatchard or Woolf-Augustinsson-Hofstee (not shown). This competitive binding between DCMU and PQ-1 suggests an identical binding site for both compounds. The possibility of two different binding

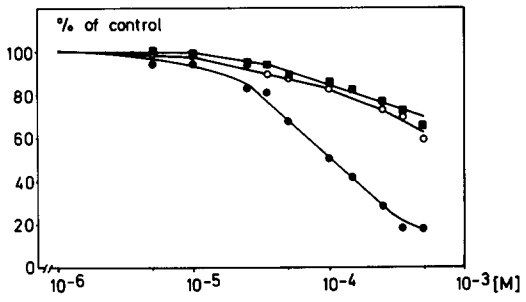

Fig. 2. Displacement of $5 \cdot 10^{-9}$ M (10 pmol) [^{3}H]DCMU by PQ-1 in control (■——■), plastoquinone-depleted (●——●) and reconstituted (○——○) thylakoid membranes.

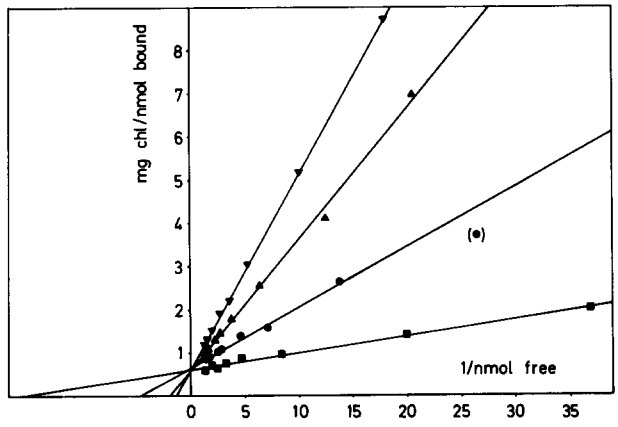

Fig. 3. Lineweaver-Burk plots for binding of [^{3}H]DCMU in plastoquinone-depleted thylakoid membranes. Control (■——■); +44 μM PQ-1 (●——●); +133 μM PQ-1 (▲——▲); +303 μM PQ-1 (▼——▼).

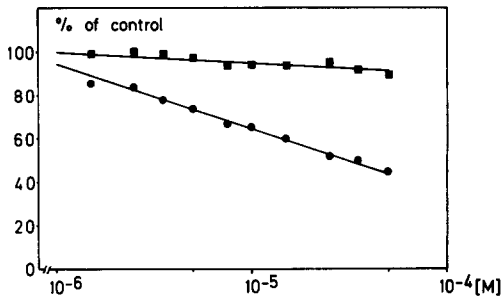

Fig. 4. Displacement of $5 \cdot 10^{-9}$ M (10 pmol) [^{3}H]DCMU by DCIP in control (■———■) and plastoquinone-depleted (●———●) thylakoid membranes.

sites, which are closely related to and mutually influence each other, however, cannot be ruled out.

Following the procedure of Tischer and Strotmann [10] and Laasch et al. [14], a binding constant $K_b = 51 \pm 19$ μM could be calculated for PQ-1.

The PS II electron acceptor DCIP in its displacement behavior towards [^{3}H]DCMU is very similar to PQ-1. There is almost no displacement of [^{3}H]DCMU by DCIP in untreated thylakoids, whereas efficient displacement is observed in plastoquinone-depleted thylakoid membranes (Fig. 4). In a series of competition experiments (Table I) again competitive interaction between DCMU and DCIP could be verified. A binding constant $K_b = 20 \pm 3$ μM was calculated for DCIP.

The competition experiments as discussed have clearly demonstrated competitive binding on the thylakoid membrane between the inhibitor DCMU and the native acceptor analogue PQ-1 and the artificial acceptor DCIP as well. However, affinities for the binding site for DCMU as compared to PQ-1 or DCIP differ by more than three orders of magnitude. This difference in affinities might explain the high efficiency of DCMU and related compounds as inhibitors of photosynthetic electron transport and hence their importance as herbicides.

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