Herbicide-binding to thylakoid membranes of a DCMU-resistant mutant of Chlamydomonas reinhardii

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The specific binding of DCMU and atrazine to the thylakoid membranes of a uniparentally inherited DCMU-resistant mutant *dr-416* of *Chlamydomonas reinhardii* was measured. Whole cells of the mutant can tolerate a 15-fold concentration of DCMU as compared to the parent strain. The same tolerance is found for the photosystem II activity of isolated thylakoid membranes. The mutant is not resistant against atrazine. In equilibrium-binding studies with [^{14}C]atrazine and unlabelled DCMU, the specific binding for atrazine was found to be identical in the mutant and in the parent strain. The competitive binding of DCMU is significantly weaker for membranes of the mutant than of the parent strain, the equilibrium dissociation constants being 2.0×10^{-7} M and 3.8×10^{-8} M, respectively.

Chlamydomonas reinhardii *Uniparental inheritance* *DCMU-resistance* *Herbicide-binding* *Thylakoid membrane*

1. INTRODUCTION

Several chemically different classes of herbicides act on the photosynthetic electron-transport chain by blocking the reducing side of photosystem II. Among them are the substituted ureas and the *s*-triazines [1], which have often been used in the study of the function of the thylakoid membrane. DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and atrazine [2-chloro-4-(2-propylamino)-6-ethylamino-*s*-triazine] are thought to have identical or at least partially overlapping sites of action [2–4]. Covalent binding of radioactively labeled azido-atrazine to thylakoid membranes of *Amaranthus* has shown a 32 kDa polypeptide of the thylakoid membrane to be responsible for the binding of the herbicide [5, 6]. In *Amaranthus* mutants resistant against atrazine, azido-atrazine no longer binds to the 32 kDa polypeptide [7]; the resistance has been found to be maternally inherited [8]. In *Spirodela*, the loss of DCMU-sensitivity of chloroplast membranes after mild trypsin treatment is correlated to a partial digestion of a 32 kDa-polypeptide which, by its biochemical and genetic character, is thought to be homologous to the atrazine-binding polypeptide [9].

In [10], a uniparentally inherited DCMU-resistant mutant of *Chlamydomonas reinhardii* was described where the photosystem II activity of isolated thylakoid membranes can be inhibited only by a significantly greater DCMU-concentration as compared to normal membranes. An increased concentratrion of DCMU for the inhibition of photosystem II activity may be due either to a loss in the number of binding sites or to a weaker specific binding of the inhibitor to the membranes, or both. The most direct information about the molecular basis of herbicide resistance can be obtained from binding measurements with radioactively labeled herbicides.

We report here on the measurements of the competitive binding of DCMU and atrazine to the thylakoid membranes of a DCMU-resistant mutant of *Chlamydomonas reinhardii*, the strain

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dr-416, which was isolated in our laboratory. Our mutant is of the type termed 'primary' [10]; i.e., it is a uniparentally inherited mutant whose membranes show a reduced affinity for DCMU.

2. MATERIALS AND METHODS

Chlamydomonas reinhardii 137c arg-8⁻mt⁺ (Cambridge Culture Collection) was used as a parent strain for mutant induction. Cultures were grown in a minimal medium [11] supplemented with 0.1 g arginine-hydrochloride/l. Mutants were induced with MNNG [1-methyl-3-nitro-1-nitrosoguanidine] as in [12] with final conc. 0.05 mg MNNG/ml. DCMU-resistant colonies were isolated on minimal medium (1.5% agar) containing 1×10^{-5} M DCMU (DuPont). Crossing procedure and tetrad analysis were done as in [13] with the arginine-autotrophic strain *C. reinhardii 137c thi 2⁻ mt⁻* (Cambridge Culture Collection). Growth rates were measured by counting cell numbers in a Coulter Particle Counter.

Photosynthetic oxygen evolution by whole cells was assayed in a Clark-type electrode (Rank Brothers). The electrode chamber contained about 2×10^7 cells suspended in 2 ml of an oxygen-depleted buffer of 10 mM Tris–citrate (pH 7.8) 60 mM $NaHCO_3$, and was illuminated with a projector lamp (10 000 lux).

For preparation of thylakoid membranes, cell suspensions (10^8 cells/ml) were sonicated in 10 mM Tricine–NaOH (pH 7.8), 50 mM NaCl. Cell debris was removed by centrifugation at $500 \times g$, the membranes were pelleted at $17000 \times g$ and resuspended to ~ 1 mg chl/ml; chlorophyll concentration was estimated spectrophotometrically as in [14].

Photosystem II activity was measured as light-dependent reduction of DCPIP [2,6-dichlorophenolindophenol] in a Beckman-25 spectrophotometer adapted for side-illumination of the sample cuvette with red actinic light (Filter Schott RG 630), 1.7 $mE \cdot s^{-1} \cdot m^{-2}$, and fitted with a 579 nm narrow band interference filter (Schott). Cuvettes contained thylakoid membranes at 0.01 mg chl/ml in 10 mM Tricine–NaOH (pH 7.8), 50 mM NaCl and 0.5 mM DCPIP. DCMU and atrazine were added from ≤ 1% ethanolic stock solutions.

Measurements of herbicide binding to thylakoids and calculations of the binding constants and of the number of specific binding sites were as in [2]. ^{14}C-Labeled atrazine with spec. act. 5.86 Ci/mol was a gift from Ciba-Geigy (Basel). Membranes (0.05 mg chl) were incubated in 0.5 ml 10 mM Tricine–NaOH (pH 7.8), 50 mM NaCl, supplemented with ^{14}C-labeled atrazine at 10^{-8}–10^{-6} M. For measuring competitive binding of DCMU and atrazine, unlabeled DCMU was added to the samples at 5×10^{-6} M.

3. RESULTS AND DISCUSSION

The mutant *dr-416* was isolated in our laboratory after MNNG-treatment of the strain *Chlamydomonas reinhardii arg 8 mt⁺* (referred to as parent strain). This mutant was 1 of 6 colonies found growing on minimal medium containing 10^{-5} M DCMU, a concentration which completely inhibits the growth of the parent strain. Fig. 1 shows the inhibition of the growth rates (a) and of the photosynthetic oxygen evolution (b) by increasing DCMU-concentrations for the parent strain

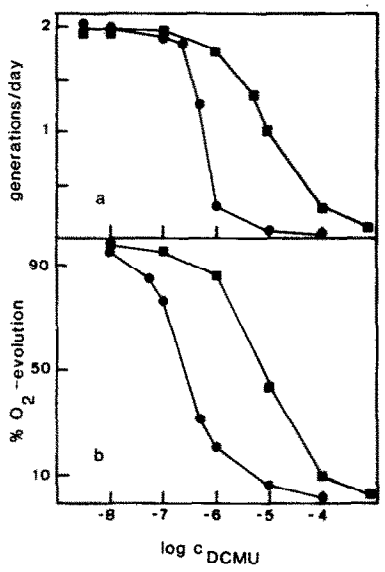

Fig. 1. (a) Growth rates in generations × day^{-1} and (b) percents of oxygen evolution by whole cells as a function of increasing DCMU-concentrations for the parent strain (—●—) and the mutant *dr-416* (—■—). Mean 100%-activities were 87.4 ± 6.8 μmol $O_2 \cdot h^{-1} \cdot (mg\ chl)^{-1}$ for the parent strain (4 expt.) and 81.3 ± 7.9 μmol $O_2 \cdot h^{-1} \cdot (mg\ chl)^{-1}$ for the mutant (2 expt). For details refer to section 2.

and the mutant. From the graphs, the half-inhibitory concentrations (I_{50}) were estimated and the values are presented in table 1. The values show that intact cells of the mutant can tolerate a 15-fold DCMU-concentration as compared to the sensitive strain.

DCMU-resistance was also reflected in isolated thylakoid membranes (fig. 2a). The light-driven DCPIP-reduction in membranes of the mutant was more resistant to DCMU than in membranes of the parent strain. The resistance factor is also about 15. As compared to intact cells, the absolute I_{50}-values were lowered by one order of magnitude, indicating that isolated thylakoid membranes of both mutant and parent strain were more susceptible to the herbicide than whole cells. The mutation in *dr-416* did not affect the sensitivity against atrazine, in contrast to DCMU. As shown in fig. 2b, the kinetics of inhibition of photosystem II activity by atrazine was identical for membranes of the parent strain and of the mutant.

Table 1

Half inhibitory concentrations (I_{50}) and equilibrium dissociation constants (K_d) of the herbicides atrazine and DCMU in the parent strain *Chlamydomonas reinhardii arg 8⁻ mt⁺* and the mutant *dr-416*

	Parent strain	Mutant *dr-416*
DCMU		
Inhibition of growth rate (I_{50})	6×10^{-7} M	1×10^{-5} M
Oxygen evolution by whole cells (I_{50})	4×10^{-7} M	6.3×10^{-6} M
DCPIP-photoreduction (I_{50})	2.0×10^{-8} M	3×10^{-7} M
Binding constant (K_d)	3.8×10^{-8} M	2.0×10^{-7} M
Atrazine		
DCPIP-photoreduction (I_{50})	1.2×10^{-7} M	1.2×10^{-7} M
Binding constant (K_d)	2.3×10^{-7} M	2.3×10^{-7}M

I_{50}-values were estimated from the graphs of the inhibitory action of the herbicides. Dissociation constants were evaluated as in [2]

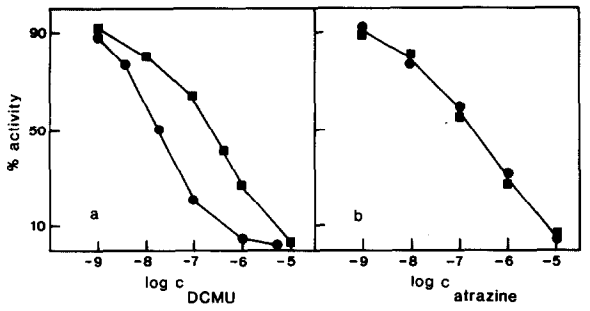

Fig. 2. Inhibition of the phostosystem II activity (assayed as DCPIP photoreduction) of thylakoid membranes of the parent strain (—●—) and the mutant *dr-416* (—■—) by various concentrations of DCMU (a) and atrazine (b): in expt. (a), 100% activities were 136 and 117 μmol DCPIP . h^{-1} . (mg chl)$^{-1}$ for the parent strain and the mutant; in expt (b), the values were 109 and 115 μmol DCPIP . h^{-1} . (mg chl)$^{-1}$, respectively. The measurements were done as in section 2.

Measurements on the equilibrium binding of ^{14}C-labeled atrazine to the membranes confirmed the latter result (fig. 3a). In a double reciprocal plot of the concentrations of free atrazine *vs*

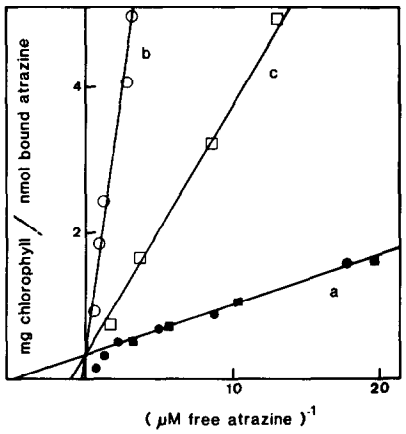

Fig. 3. Double reciprocal plots of the concentrations of free [^{14}C]atrazine *vs* bound [^{14}C]atrazine after equilibration with thylakoid membranes. (a) Binding of atrazine to thylakoids of the parent strain (—●—) and the mutant *dr-416* (—■—); no DCMU present. (b) Binding of atrazine to thylakoids of the parent strain (—○—) in the presence of 5×10^{-6} M DCMU. (c) Binding of atrazine to thylakoids of the mutant *dr-416* (—□—) in the presence of 5×10^{-6} M DCMU. The values for the concentration of specific binding sites and for the equilibrium dissociation constants are given in table 1. For experimental details see section 2.

bound atrizine, the values for the mutant and the parent strain lie on the same straight line. From the intercepts with the axes the equilibrium dissociation constant of membrane-bound atrazine (referred to as binding constant) and the concentration of specific binding sites can be determined [2,15]. The resulting values of 2.3×10^{-7} M for the binding constant of atrazine and of one binding site 400 chl molecules are in close accordance to the respective values reported for other organisms [2,4].

In the presence of DCMU, less atrazine remained bound to the thylakoid membranes (fig. 3b, c). The identical intercepts of all the graphs with the ordinate demonstrate a competitive binding for the two herbicides. Therefore, by assuming identical binding sites, we could calculate the equilibrium dissociation constant for DCMU [2]. We obtained values of 3.8×10^{-8} M for the sensitive parent strain and 2.0×10^{-7} M for the resistant mutant *dr-416*. The binding constant for the sensitive strain is close to the value for *Chlamydomonas* reported in [15], where measurements were made directly with labeled DCMU. The same authors also gave values for unspecific binding of DCMU to the thylakoid membrane. Our measurements show that the binding of this herbicide in the mutant *dr-416* is stronger than the unspecific binding by about one order of magnitude. Therefore, we assume that DCMU-binding in the mutant still is a specific process, although with a significantly reduced affinity for the inhibitor.

The DCMU-resistance in our mutant *dr-416* is persistent in the absence of DCMU and is uniparentally inherited. In tetrad analysis of crosses of the mutant with an arginine-autotrophic strain all the progeny of the 20 isolated tetrads were resistant against DCMU, while the allele arg^+/arg^- was distributed in a Mendelian 2:2 fashion. Since the mutation affects chloroplast membranes, one can assume it to be located in the chloroplast genome. The mutant *dr-416* therefore belongs to the type termed 'primary' [10] and shows some similarity to their mutant *Dr2*. In both mutants, almost the same resistance factor for DCMU was found, in spite of the different methods for its measurement. However, the two DCMU-resistant mutants seem to differ in their sensitivity against atrazine and in the absolute value of the binding constant for DCMU. A detailed genetic analysis with mapping of the gene locus for DCMU-resistance would therefore be of considerable interest. Furthermore, the identification of the molecular alteration in the thylakoid membrane would be very helpful to our understanding of herbicide binding and action and of the regulation of the electron transport at photosystem II.

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