Biochimica et Biophysica Acta, 681 (1982) 21–31
Elsevier Biomedical Press

BBA 41091

THE DICHLOROPHENYLDIMETHYLUREA-BINDING SITE IN THYLAKOIDS OF *CHLAMYDOMONAS REINHARDII*

ROLE OF PHOTOSYSTEM II REACTION CENTER AND PHOSPHORYLATION OF THE 32–35 KILODALTON POLYPEPTIDE IN THE FORMATION OF THE HIGH-AFFINITY BINDING SITE

SUSANA SHOCHAT, GEOFFREY C. OWENS, PIERRE HUBERT * and ITZHAK OHAD

Department of Biological Chemistry, The Hebrew University of Jerusalem, Institute of Life Sciences, 91904 Jerusalem (Israel)

(Received February 11th, 1982)

Key words: Photosystem II; Reaction center; Herbicide; Membrane protein; Phosphorylation; Dichlorophenyldimethylurea; (C. reinhardii)

Binding of ^{3}H-labeled dichlorophenyldimethylurea (DCMU) to thylakoids from two conditional mutants of *Chlamydomonas reinhardii*, having normal or altered polypeptide composition, was studied. Membranes could be obtained having polypeptide(s) participating in the formation of Photosystem II (PS II) reaction center (44–54 kDa) and the 32–35 kDa polypeptide involved in herbicide binding, having only one of these polypeptides or none. The 32–35 kDa polypeptide in *Chlamydomonas* is phosphorylated and its phosphorylation state is affected by membrane-bound kinase and phosphatase. The latter preferentially removes phosphate from the 32–35 kDa polypeptide in vitro. Normal membranes possess approx. 1 DCMU-binding site/600 chlorophyll molecules with a binding constant of $2–6 \cdot 10^{-8}$ M (high-affinity site). Alteration of the PS II reaction center, without loss of the 32–35 kDa polypeptide, reduces significantly the number of high-affinity binding sites and increases the value of the binding costant to about 10^{-7} M (low-affinity site). A similar situation is obtained following in vitro dephosphorylation of the 32 kDa polypeptide. A reduction in the number of high-affinity sites and an increase in the binding constant are also observed in membranes having an active PS II reaction center but depleted of the 32–35 kDa polypeptide. The number of high-affinity sites increases following insertion of the 32–35 kDa polypeptide into such membranes in vivo. It is concluded that the formation of the high-affinity DCMU-binding site requires the presence of a phosphorylated 32–35 kDa polypeptide and a functional organization of the PS II reaction center.

Introduction

In recent years, increasing efforts have been made to elucidate the mechanism of herbicides' action known to inhibit photosynthetic electron flow at the reducing site of PS II, and attempts have been made to identify the polypeptides forming the binding site of herbicides [1–18]. We have shown before that mild trypsinization of isolated photosynheic membranes obtained from *Chlamydomonas reinhardii* does not inactivate the reaction centers of PS II and PS I. However, PS II activity of the trypsinized membranes was not inhibited by DCMU [19,20]. Examination of the polypeptide electrophoretic pattern of trypsin-treated membranes showed that only a few polypeptides were

* Present address: Centre de Neurochimie du C.N.R.S., 11, rue Humann, 67085 Strasbourg, France.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; Chl, chlorophyll; PS, photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHCP, light-harvesting chlorophyll *a*/*b*-protein complex; atrazine, 2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine; dinoseb, 2-(*sec*-butyl)-4,6-dinitrophenol; LDS, lithium dodecyl sulfate; metribuzine (sencor), 4-amino-6-(*tert*-butyl)-3-(methylthio)-1,2,4-triazine-5-(4H)-one.

affected, including components of the chlorophyll *a/b*-protein complex (LHCP) and polypeptides in the molecular mass range 32–35 kDa [2,19]. Similar results were obtained with higher plants' chloroplasts [1,4,10,11,21], and it was assumed that the latter polypeptides could be involved in the formation of an intermediary electron carrier between PS II and PS I, and possibly also the binding site of DCMU.

A similar mechanism for herbicide action was demonstrated from competition experiments for a variety of PS II inhibitors [5], and several reports indicated that trypsin digestion of the herbicide-binding polypeptide resulted in loss of sensitivity toward chemically unrelated PS II inhibitors such as DCMU, atrazine or metribuzine [4,12].

Based on photoaffinity labeling studies using azido-atrazine, the 32 kDa polypeptide was identified as the herbicide-binding site in higher plants [8,10]. Furthermore, this herbicide did not bind to the 32 kDa polypeptide in thylakoids of the herbicide-resistant *Amaranthus hybridus* [8]. These and previous findings will obviously prompt workers in this field to attempt the identification, isolation and cloning of the chloroplast resistant gene which might serve for engineering of herbicide-resistant crops. The economic significance of such an achievement does not need further emphasis here. However, the possibility should be considered that more than one polypeptide participates in the formation of the above-mentioned herbicide-binding site(s): this, in view of results obtained from studies of the inhibition-release kinetics of trypsinized chloroplasts showing different specific subreceptor sites [15] and differences in the inhibition of trypsinized membranes toward various classes of inhibitors [4,12], as well as the persistence of sensitivity toward DCMU in atrazine-resistant mutants of *A. hybridus* [14] and *Senescio vulgaris* [2], or toward simazine in DCMU-resistant mutants of *Chlamydomonas* [13]. Moreover, selective extraction of the 32–34 kDa polypeptide from isolated active PS II particles abolished the inhibitory effect of atrazine, without affecting the inhibition of PS II activity by DCMU or dinoseb [10]. Photoaffinity labeling of chloroplast membranes by azido-dinoseb showed that the herbicide binds to a 41 kDa polypeptide [11].

These results could be explained if one supposes that the binding site is formed by the participation of several polypeptides able to assume more than one configuration and recognize a variety of chemical groups. Thus, it appears to us that the search for additional thylakoid polypeptides acting as binders or modifiers of the properties of the herbicide-binding site or domain might provide useful information.

The approach taken in this work was to determine the number of binding sites and their affinity for DCMU in a variety of membranes having altered polypeptide patterns, and to try to establish a correlation between the binding of DCMU and the presence of specific thylakoid polypeptides.

Recent studies have demonstrated that several thylakoid polypeptides forming the LHCP can be phosphorylated in vitro, using intact chloroplasts and ^{32}P, or isolated thylakoids and [γ-^{32}P]ATP [22–26]. As a result of a study on thylakoid polypeptide phosphorylation in intact *C. reinhardii* cells and isolated thylakoids, it was found that in addition to the LHCP polypeptides, phosphorylation occurred both in vitro and in vivo, also for the trypsin-sensitive polypeptides of 32–35 kDa [27,28]. Thus, the question arose as to whether phosphorylation of these polypeptides might play a role in the action of herbicides.

The results obtained so far indicate that in addition to the requirement for the presence of 32–35 kDa polypeptides, DCMU binding is also affected by the presence of polypeptides in the molecular mass range 44–54 kDa required for the formation of the PS II reaction center [29,30]. Incubation of chloroplast membranes in vitro under conditions of dephosphorylation induces changes in the affinity constant for DCMU binding. Part of the results presented in this work has been published as a preliminary communication [31].

Materials and Methods

Cultivation and properties of the mutant cells

Two *C. reinhardii* mutants were used: y-1 and T_{44}. The y-1 mutant has been described in detail previously [32]. Briefly, it is characterized by its inability to synthesize chlorophyll when grown in the dark. Dark-grown cells contain only remnants

of the photosynthetic membranes which have components of the PS II and PS I reaction centers [32,33] but do not have an organized LHCP nor the polypeptides in the molecular mass range 32–35 kDa. Upon exposure to light under nondividing conditions (greening), chlorophyll is synthesized and normal photosynthetic membranes are formed. Synthesis of 70S translates can be inhibited by chloramphenicol during the greening process, resulting in the formation of membranes possessing a normal LHCP but lacking PS II and PS I reaction centers. These can be subsequently formed, if 70S translation is resumed (repair) [34–36]. The repair process can be carried out under incubation conditions which might allow or prevent the synthesis of chlorophyll and cytoplasmic translates, as well as the light-dependent synthesis of 70S translates participating in the formation of the thylakoid membranes such as polypeptides of 32–35 kDa [34,35]. However, as demonstrated previously, the synthesis of 70S translates required for the formation of PS II and PS I reaction centers is light independent [32].

The second mutant, T_{44}, was isolated in our laboratory as a spontaneous mutation from a culture of the T_4 temperature-sensitive mutant described by Chua and Bennoun [29]. The T_{44} mutant, in addition to its inability to form an active PS II complex when grown at 37°C, as originally reported for the T_4 mutant, has also lost the ability to synthesize chlorophyll in the dark when grown at the permissive temperature (25–36°C).

Both mutants were grown on a mineral medium containing acetate as the sole carbon source [37]. Greening of dark-grown y-1 mutant cells in the presence or absence of chloramphenicol and repair experiments were carried out as described previously [34–36]. When used, chloramphenicol was added to a final concentration of 200 μg/ml and cycloheximide, 1–2 μg/ml.

Prepation of membranes and assays of various activities

For measurements of photosynthetic activity and DCMU-binding experiments, thylakoid membranes were prepared as described previously [34]. Phosphorylation and dephosphorylation experiments were carried out with membranes prepared by a slight modification [28] of the procedure reported by De Petrocellis et al. [38].

Measurements of photosynthetic activities were carried out spectrophotometrically or polarographically, using H_2O or diphenylcarbazide as an electron donor and DCIP as an electron acceptor for PS II [33], and reduced DCIP and methyl viologen as electron donor and acceptor for PS I, respectively [39]. Measurements of variable fluorescence were carried out using the apparatus described previously [33]. Fluorescence emission spectra at 77 K were carried out as described by Gershoni and Ohad [40]. Analysis of the thylakoid polypeptide pattern was carried out by LDS-polyacrylamide gel electrophoresis, as described by Chua [41]. Phosphorylation of membrane polypeptides was carried out in vivo by incubating the cells in the presence of 10^{-5} M phosphate (500 μCi ^{32}P/mmol) or in vitro by incubating chloroplast membranes with [γ-^{32}P]ATP (200 μCi/mmol) (obtained from Nuclear Research Center, Beer Sheba, Israel). Detection of phosphorylated polypeptides was carried out by autoradiography of dried slab gels using the standard procedures.

For DCMU-binding studies, [^3H]DCMU was prepared by tritiation of DCMU obtained from DuPont as a gift. Tritiation and purification were carried out by the Nuclear Research Center, Beer Sheba, Israel. The specific radioactivity obtained was 3.54 Ci/mmol. Binding of DCMU was assessed by incubating isolated chloroplast membranes (50–100 μg Chl) at 25°C for 3 min in a buffer containing 30 mM Tris-HCl, pH 8, and 30 mM KCl. The incubation mixture was then centrifuged to sediment the membranes completely (10000 × *g* for 10 min) and the radioactivity of the supernatant was measured. The experimental data are presented as double-reciprocal plots of free vs. bound DCMU, from which the number of sites and affinity constants were determined following linear regression analysis of the curves [5].

Results

Binding of DCMU to chloroplast membranes obtianed from light-grown cells (y-1): Effect of trypsinization

Membranes of light-grown y-1 cells are photosynthetically active and exhibit a normal polypeptide pattern (Fig. 1). DCMU-binding experi-

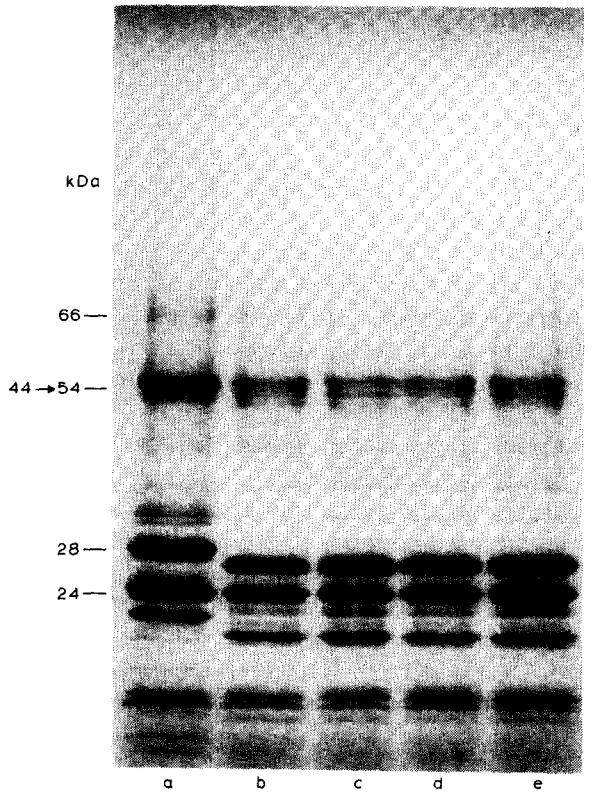
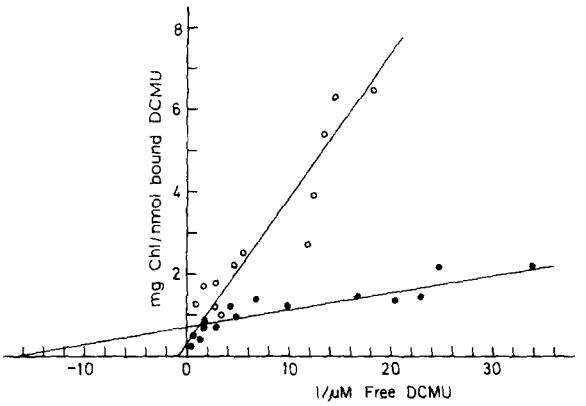


Fig. 1. LDS-polyacrylamide gel electrophoresis of trypsin-treated membranes obtained from light-grown *Chlamydomonas reinhardii* y-1 cells. Untreated membranes (a), membranes treated with 50 or 100 μg/ml trypsin in the absence or presence of 10^{-5} M DCMU (b, c and d, e, respectively). Incubation was carried out at 25°C for 15 min at a membrane concentration equivalent to 0.5 mg Chl/ml.

ments using such membranes showed that about 1 nmol DCMU is bound per 0.580 mg Chl, or about 1 binding site/600 Chl molecules. The affinity binding constant was found to be $5.9 \cdot 10^{-8}$ M (Fig. 2). Trypsin treatment of these membranes, in the presence or absence of 10^{-5} M DCMU, resulted in the loss of polypeptides of 32–35 kDa and change in the electrophoretic mobility of the LHCP polypeptides (Fig. 1). The number of DCMU molecules bound by trypsinized membranes doubled (approx. 1 DCMU molecule/300 Chl molecules) but the affinity decreased about 20-fold ($1.2 \cdot 10^{-6}$ M) (Fig. 2). These results indicate that the high-affinity DCMU-binding site was lost after trypsinization and removal of the 32–35 kDa polypeptides, while nonspecific binding of lower affinity became predominant.



. Fig. 2. Double-reciprocal plot of [^{3}H]DCMU binding to trypsin-treated thylakoid membranes of light-grown *Chlamydomonas reinhardii* y-1 cells. Incubation was carried out for 30 min at 10°C; trypsin concentration was 150 μg/ml and Chl concentration 100 μg/ml. Values derived from intercepts: (●) control, 0.73 mg Chl/nmol DCMU; $K_b = 5.9 \cdot 10^{-8}$ M; (○) trypsin-treated membranes, 0.33 mg Chl/nmol DCMU; $K_b = 1.2 \cdot 10^{-6}$ M.

Binding of DCMU to chloroplast membranes obtained from the T_{44} mutant cells grown at permissive and nonpermissive temperatures

The electrophoretic pattern of thylakoid membranes of T_{44} cells grown at permissive (25–27°C) or nonpermissive (37°C) temperatures is shown in Fig. 3. It can be seen that only polypeptides in the molecular mass range 44–54 kDa are missing in membranes of 37°C-grown cells, while the 32–35 kDa region is identical to that of membranes obtained from 25°C-grown cells, as was also found in the temperature-sensitive T_4 mutant (see also Ref. 29). About 1 molecule of DCMU is bound per 815 Chl molecules in the 25°C-grown membranes, as compared with 1700 Chl molecules in the membranes of 37°C-grown cells. At the same time, the affinity binding constant increases about 8-fold (from $1.6 \cdot 10^{-8}$ to $1.3 \cdot 10^{-7}$ M) (Fig. 4). The loss of the polypeptides in the molecular mass range 44–54 kDa is accompanied by complete loss of variable fluorescence (Fig. 5) and alteration of the fluorescence emission spectrum at 77 K (Fig. 6). Loss of variable fluorescence is considered to represent loss or disorganization of PS II reaction centers, and was reported in *Chlamydomonas* mutants lacking PS II [29]. Thus, disorganization of the reaction center of PS II causes a loss of the high-affinity sites, despite the fact that the poly-

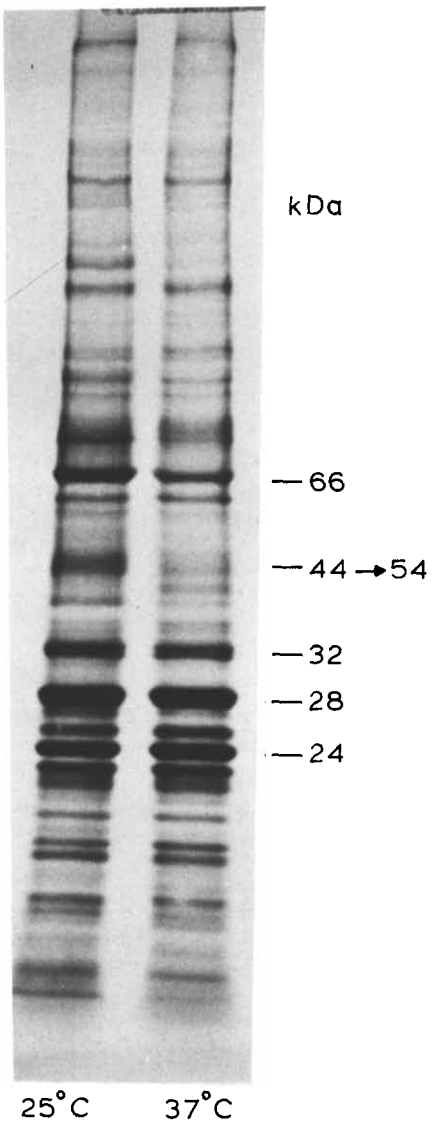


Fig. 3. Autoradiogram of the electrophoretic pattern of thylakoid polypeptides of *Chlamydomonas reinhardii* T_{44} mutant. Cells were grown for two to three generations at 25°C or at 37°C in the presence of [^{14}C]acetate (1 μCi/μmol).

peptides of the 32–35 kDa region are present in the membranes of the 37°C-grown cells.

Binding of DCMU to membranes formed in absence of 70S translation activity

The electrophoretic pattern of membranes formed during greening of dark-grown *C. reinhardii* y-1 is indistinguishable from that of membranes formed in light-grown cells. However, cells

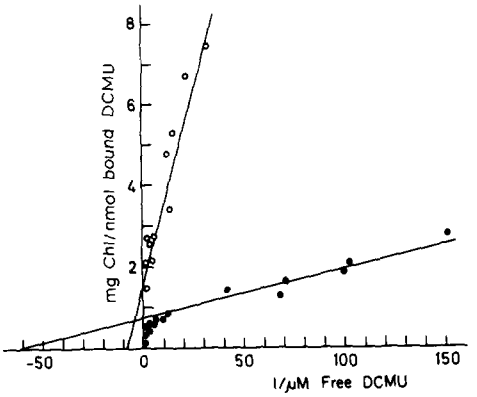


Fig. 4. Double-reciprocal plot of [^{3}H]DCMU binding to thylakoid membranes of *Chlamydomonas reinhardii* T_{44} cells grown at permissive (25°C) (●) and nonpermissive (37°C) (○) temperatures. Values derived from intercepts: T_{44}, 25°C: 0.77 mg Chl/nmol DCMU; $K_b = 1.6 \cdot 10^{-8}$ M. T_{44}, 37°C: 1.6 mg Chl/nmol DCMU; $K_b = 1.3 \cdot 10^{-7}$ M.

greening in the presence of chloramphenicol synthesize the LHCP polypeptides but not those in the molecular mass range 32 and 44–68 kDa (Fig. 7). Membranes formed in the presence of chloramphenicol appear to bind 1 DCMU molecule/153 Chl molecules (Table I), viz., about 4-times more as compared with normal membranes. However, the affinity of these sites is about 12-times lower than that of normal photosynthetic membranes.

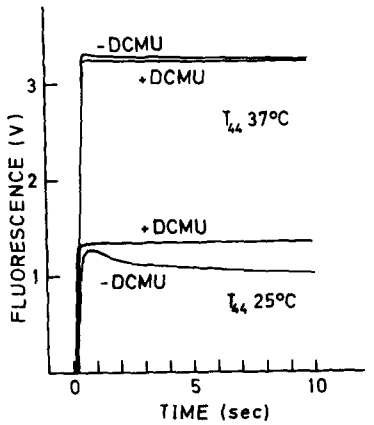


Fig. 5. Recorder traces of fluorescence induction kinetics obtained from thylakoid membranes of *Chlamydomonas reinhardii* T_{44} grown at permissive (25°C) and at nonpermissive (37°C) temperatures in the absence and presence of DCMU (10^{-5} M). Data were obtained at equal light intensities and chlorophyll concentration.

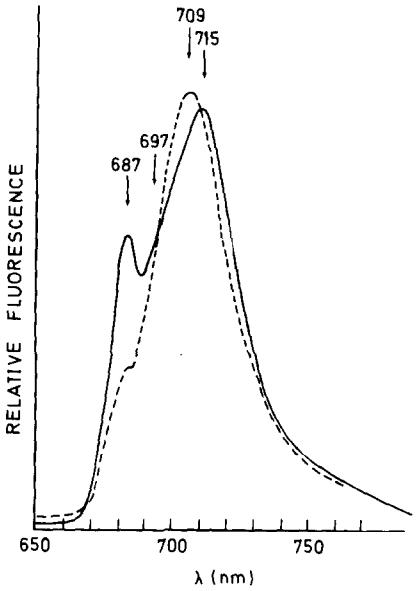


Fig. 6. Fluorescence spectra at 77 K of thylakoid membranes obtained from *Chlamydomonas reinhardii* T_{44}, cells grown at permissive (25°C) (———) and nonpermissive (37°C) (- - - - -) temperatures. The emission was normalized to the highest fluorescence peak. Excitation was at 380–620 nm, using a Corning 4-96 blue filter.

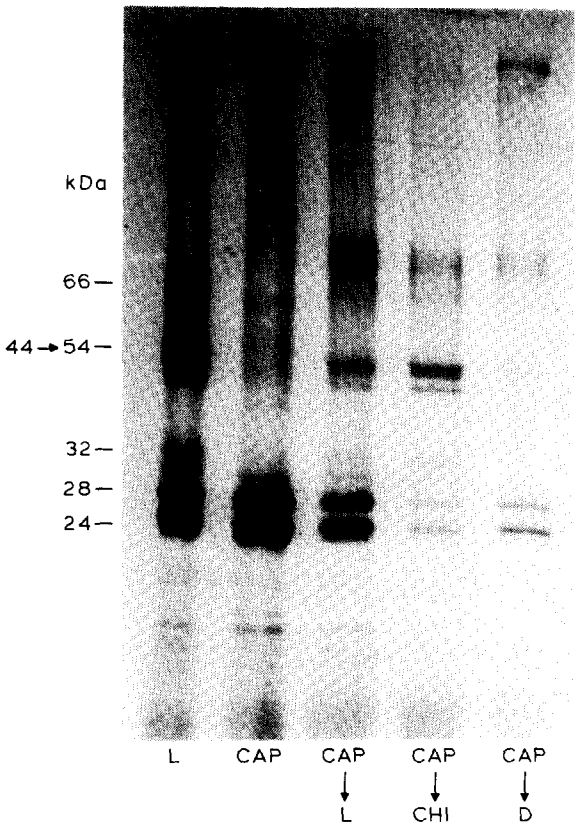


Fig. 7. Autoradiogram of the polypeptide pattern of thylakoid memranes of *Chlamydomonas reinhardii* y-1 cells greened in the light(L) or in presence of chloramphenicol (CAP) and further incubated in the light (L) or dark (D) in the presence or absence of cycloheximide (CHI). Dark-grown cells (Chl concentration 0.8 $\mu g/10^7$ cells) were incubated in the light in the presence of chloramphenicol (200 μg/ml) for 6 h (CAP; Chl concentration 2.8 $\mu g/10^7$ cells). The cells were washed free of chloramphenicol and further incubated in the light (CAP→L; Chl concentration 7.2 $\mu g/10^7$ cells), in the dark (CAP→D; Chl concentration 2.8 $\mu g/10^7$ cells), and in the light in the presence of cycloheximide (CAP→CHI; Chl concentration 3.8 $\mu g/10^7$ cells). All incubations were carried out in the presence of [^{14}C]acetate (1.5 μCi/μmol). PS II and PS I activities (μmol DCIP reduced or oxygen consumed/mg Chl per h) were: CAP, 24 and 104; CAP→L, 320 and 630; CAP→D, 80 and 366; CAP→L+CHI, 150 and 300.

The membranes formed in the presence of chloramphenicol are highly fluorescent [35], do not exhibit the normal 714 nm fluorescence peak at 77 K, but have a high fluorescence emission at 705–708 nm [42,43] and are photosynthetically inactive. The organization and activity of these membranes can be repaired, if the cells are washed free of chloramphenicol and further incubated in the light, dark, or light with the addition of cycloheximide which prevents 80S translation and chlorophyll synthesis [32].

In order to identify the polypeptides synthesized during the repair process, the cells were incubated in the presence of [^{14}C]acetate as a precursor for synthesis of all cell constituents [34]. Membranes were isolated from cells repaired under various conditions, the polypeptides resolved by LDS-polyacrylamide gel electrophoresis, and the newly synthesized polypeptides identified by autoradiography of the dried slab gel. Results of such an experiment are shown in Fig. 7. It is evident that photosynthetic activity was reestablished in all cases in which polypeptides in the molecular mass ranges 44–54 and 66–68 kDa were synthesized.

Only polypeptides synthesized by 70S ribosomes were radioactively labeled in the cells incubated in the presence of cycloheximide, including the above-mentioned ones, as well as polypeptides in the molecular mass range 32–35 kDa.

TABLE I

BINDING OF DCMU TO *CHLAMYDOMONAS REINHARDII* THYLAKOIDS WITH ALTERED POLYPEPTIDE COMPOSITION AND PHOTOSYNTHETIC ACTIVITIES

n.d., not determined. The ratio Chl/DCMU was calculated using 950 as the molecular mass of chlorophyll.

Expt. No.	Cell strain and culture conditions	μg Chl/10^7 cells	Photosynthetic activity		Membrane polypeptide molecular mass range (kDa)			μmol Chl/ μmol DCMU	K_b (M) ($\times 10^{-8}$)
			PS II	DCMU sensitive	44–54	32–35	14–18		
1 (a)	y-1, light	30–50	+	+	+	+	+	550–770	4.7–5.9
(b)	y-1, light, trypsinized	30–50	+	–	+	–	+	350	120.0
2 (a)	T_{44}, 25°C, light	30–50	+	+	+	+	+	815	1.6
(b)	T_{44}, 37°C, light	20–30	–	n.d.	–	+	+	1700	13.0
3 (a)	y-1, dark → light	10–14	+	+	+	+	+	350	12.0
(b)	y-1, dark → light + chloramphenicol	7–10	–	n.d.	–	–	+	153	71.0
	repair, dark	7–10	+	+	+	–	–	261	21.0
	repair, light + cycloheximide	8–11	+	+	+	+	–	316	3.2
	repair, light	12–16	+	+	+	+	+	322	8.4

These polypeptides were not synthesized in dark-incubated cells (Fig. 7). The Chl *a/b*-binding polypeptides of cytoplasmic origin [34] were synthesized in cells incubated in the light, in which a large amount of chlorophyll was also synthesized. These polypeptides were only slightly labeled in dark-incubated cells (Fig. 7). Furthermore, polypeptides in the molecular mass range 14–18 kDa were synthesized only in cells incubated in the light in which cytoplasmic translation was not inhibited.

Binding of DCMU to membranes obtained from these cells is shown in Table I. In all membranes about 1 DCMU molecule is bound/300 Chl molecules, as in membranes formed during greening of dark-grown y-1 cells in the absence of inhibitors. Thus, a decrease in the number of binding sites relative to chlorophyll was observed in all cases in which photosynthetic activity was reestablished during the repair process. At the same time, the affinity for DCMU binding increased about 8-fold for membranes of light-incubated cells, about 22-fold for those repaired in the presence of cycloheximide in which no chlorophyll was synthesized, and only about 3-fold for membranes obtained from dark-incubated cells. Hence, it appears that the rise in binding affinity correlates with the synthesis of 70S translates in the 32–35 kDa molecular mass range.

Effect of dephosphorylation of membrane polypeptides on DCMU binding

Several thylakoid polypeptides in *C. reinhardii* y-1 were found to be phosphorylated in vivo. Phosphorylation of membrane polypeptides can also be demonstrated in vitro by incubating isolated membranes with [γ-^{32}P]ATP. The phosphorylated polypeptides include the Chl *a/b*-binding polypeptides as well as polypeptides in the molecular mass ranges of about 12–20 and 32–35 kDa (Fig. 8). In addition, it has also been demonstrated that a protein phosphatase able to dephosphorylate these polypeptides is active both in vivo and in vitro [28]. The activity of the membrane-bound protein phosphatase in vitro appears to be higher toward the 32–35 kDa phosphorylated polypeptides (Fig. 8). However, dephosphorylation in vitro is not complete under the assay conditions.

Since polypeptides of this molecular mass (32–35 kDa) appear to be involved in the formation of DCMU-binding sites, it was of interest to find out whether their dephosphorylation might affect

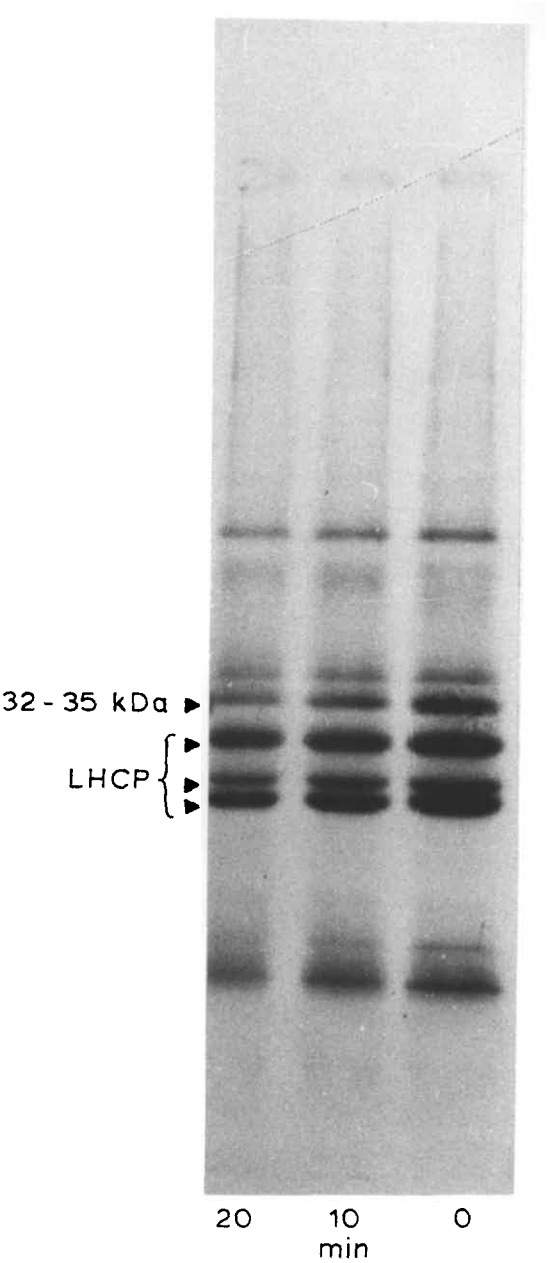


Fig. 8. Autoradiogram showing in vitro dephosphorylation of thylakoid membranes obtained from light-grown *Chlamydomonas reinhardii* y-1 cells. Isolated thylakoids were labeled in the light with [γ-^{32}P]ATP in the presence of 10 mM $MgCl_2$, sedimented, resuspended to the same protein concentration (3 μg/μl), and incubated for 10 and 20 min at 25°C in the presence of 10 mM $MgCl_2$, 0.01 mM $ZnCl_2$ and 2 mM benzamidine.

TABLE II

EFFECT OF IN VITRO DEPHOSPHORYLATION OF THYLAKOID PHOSPHOPROTEINS ON DCMU BINDING

Thylakoids obtained from ligth-grown *Chlamydomonas* y-1 cells were incubated in vitro under the same experimental conditions as in Fig. 8 but ATP was omitted. Benzamidine was added to all incubation mixtures; sodium molybdate concentration was 20 mM; values of K_b, and the ratio of DCMU bound relative to chlorophyll were determined as described in Materials and Methods.

Time of incubation (min)	K_b (M)($\times 10^{-8}$)	μmol Chl/μmol DCMU bound
0	2	730
15	3	1160
30	8	1160
30+sodium molybdate	3.5	840

DCMU binding. Results of such an experiment are shown in Table II. Following incubation of isolated membranes for 15 and 30 min at 25°C, in the absence of ATP, a decrease in DCMU binding is observed from 1 DCMU molecule/730 Chl molecules to about 1 DCMU molecule/1160 Chl molecules. The binding affinity decreases about 4-fold from $2 \cdot 10^{-8}$ to about $8 \cdot 10^{-8}$ M. These changes could not be due to partial proteolysis of the 32–35 kDa polypeptides by membrane-bound proteases [44], as the protease inhibitor benzamidine was added to the incubation mixtures and no change in the staining density of the 32–35 kDa region was observed. Furthermore, addition of molybdate which partially inhibited the dephosphorylation also inhibited the increase in the DCMU-binding constant (Table II).

Discussion

The results obtained in this work, summarized in Tables I and II, indicate that in photosynthetically active membranes of both y-1 and T_{44} mutants of *C. reinhardii*, 1 DCMU binding site is present for 520–770 Chl molecules, having an affinity constant of approx. $5 \cdot 10^{-8}$ M. These values are in agreement with data obtained from photosynthetic membranes of higher plants [2,5]. The variation observed in the ratio Chl/DCMU from 520 to 770 could be ascribed to a normal variation found in the ratio antennae Chl/reaction centers, which might be due to changes in growth conditions of *Chlamydomonas* cells, especially toward the end of the logarithmic phase of growth. Similarly, changes in the ratio antennae Chl/electron-transfer chains could account for the large increase in DCMU-binding sites per chlorophyll. The affinity constant in the range 10^{-8} M can be considered as specific for the high-affinity DCMU-binding site present in normal membranes. Values of K_b in the range of approx. 10^{-6} M could represent nonspecific binding which could be due to a partitioning of DCMU in the hydrophobic phase of the membrane. Observed values of the binding constant intermediate between 10^{-7} and 10^{-8} M could arise from binding to a specific but altered or low-affinity site due to changes in the polypeptide composition of the membranes. Trypsin digestion of higher plants' chloroplast membranes reduced the number of sites about 4-fold, while the affinity constant increased only by a factor of about two [7,11]. This could indicate mild proteolysis which did not remove all of the sites, the residual sites remaining practically unaffected. On the other hand, in our trypsinization experiments, proteolysis was probably more extensive, removing all of the DCMU-binding sites. Under these conditions, only the nonspecific binding was measured. Consequently, the calculated ratio Chl/DCMU only reflects the presence of a high number of nonspecific sites with low affinity.

The experimental system utilized in this work allowed us to obtain membranes lacking polypeptides in the molecular mass ranges 44–54 kDa, which were shown to be required for the formation of an active PS II reaction center [29,30,34], 32–35 kDa, shown to be involved in DCMU binding [1–11], and 14–18 kDa, which so far seem to be involved in the organization of the PS I antennae [45].

The absence of the 32–35 and 44–54 kDa polypeptides (and thus loss of PS II reaction center activity) causes the loss of the high- and low-affinity binding sites and an increase in DCMU binding, which is most probably nonspecific. Reintegration of the 44–54 kDa polypeptides during the repair process reestablishes PS II activity. In this case an intermediate situation is obtained, characterized by a reduction in the binding rela-

tive to chlorophyll and increase in the affinity for DCMU binding to an intermediate value between the nonspecific and high-affinity values. Addition of both types of polypeptides, in the 44–54 and 32–35 kDa ranges, restores the high-affinity binding completely. At the same time, the nonspecific binding contributes less to the total amount of DCMU bound relative to chlorophyll and, therefore, the value of this ratio decreases. On the other hand, depletion of the polypeptides of PS II reaction centers from membranes of T_{44} cells, still containing the 32–35 kDa polypeptides, alters the affinity for DCMU binding but reduces the number of binding sites by only about a half. These results could be explained if one considers that T_{44} cells cannot grow indefinitely at 37°C. PS II-deficient cells were obtained by transferring a 25°C-grown culture to 37°C and allowing the cells to divide two to four times. Thus, 37°C-grown T_{44} cells could still contain approx. 25% of the reaction center polypeptides and correlated amounts of normally organized DCMU-binding sites. The inverse situation, alteration of the 32–35 kDa polypeptides, either by digestion of the polypeptide chain exposed on the membrane surface or dephosphorylation, substantially reduces the affinity for DCMU binding and discloses the presence of nonspecific binding of lower affinity.

The absence of the low molecular mass polypeptides (14–18 kDa) does not seem to affect the properties of the DCMU-binding sites in *Chlamydomonas.*

The results presented in this work indicate that the formation of the high-affinity binding site for DCMU in *Chlamydomonas* requires participation of two types of polypeptides: (1) the 32–35 kDa polypeptides to which herbicides appear to bind specifically and (2) polypeptides participating in the formation of the PS II reaction centers which seem to affect the conformation of the former polypeptides in such a way as to give rise to the high-affinity site. Apparently, DCMU can still bind and inhibit electron flow of the PS II reaction center, even in the absence of the 32–35 kDa polypeptides. However, in this case the affinity is significantly reduced. These results are in agreement with data obtained by Mullet and Arntzen [10] showing that atrazine does not inhibit the activity of isolated PS II particles depleted in the 32–35 kDa polypeptides, whereas DCMU (10^{-4} M) still inhibits the activity.

The possibility that the high-affinity herbicide-binding site is formed by the interaction of several polypeptides able to assume several configurations with various specificities has been considered by Renger [16] and by Arntzen et al. [14]. Our results are consistent with such a model and with the view that the normal organization of the PS II complex in *Chlamydomonas* requires the presence of all polypeptides involved, including polypeptides 5, 6, 12, and the 32–35 kDa polypeptides, D_1 (according to the nomenclature of Chua [41]), to which the herbicides seem to bind. The state or organization of this complex appears to be affected by the degree of phosphorylation of its components. Recent results obtained in our laboratory showed that several thylakoid polypeptides associated with PS II in *Chlamydomonas* are indeed continuously phosphorylated in vivo, independent of illumination and DCMU-sensitive electron flow [28].

The 32 kDa polypeptide was found to be synthesized in both atrazine-resistant and -sensitive *Solanum, Chenopodium* and *Brassica* [21]. On the other hand, a slight change in the electrophoretic mobility of polypeptides of 22 and 24 kDa has been reported to occur in an atrazine-resistant *Amaranthus* [14]. Discrete changes in the primary structure of a polypeptide might account for changes in its ability to bind herbicides but might not be sufficient to alter its electrophoretic mobility in the presence of sodium dodecyl sulfate. In view of previous and present results, the possibility should be considered that the ability to bind various herbicides might also be affected by genetic modifications of the PS II reaction center polypeptides, which might not necessarily cause inhibition of electron flow.

Acknowledgments

This work was supported by a BARD grant No. 79-80. G.C. O. is the recipient of a NATO-SERC Overseas Studentship award.

References

1 Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300
2 Pfister, K., Radosevich, S.R. and Arntzen, C.J. (1979) Plant Physiol. 64, 995–999

3 Steinback, K.E., Burke, J.J. and Arntzen, C.J. (1979) Arch. Biochem. Biophys. 195, 546–557
4 Böger, P. and Kunert, K.J. (1979) Z. Naturforsch. 34C, 1015–1020
5 Tischer, W. and Strotmann, H. (1979) Z. Naturforsch. 34C, 992–995
6 Izawa, S. and Good, N.E. (1965) Biochim. Biophys. Acta 102, 20–38
7 Pfister, K. (1981) in Proceedings of the 5th International Congress on Photosynthesis Research (Akoyunoglou, G., ed.), vol. 6, pp. 535–604, Balaban International Science Services, Philadelphia
8 Pfister, K., Steinback, K.E., Gardner, G. and Arntzen, C.J. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 981–985
9 Croze, E., Kelly, M. and Horton, P. (1979) FEBS Lett. 103, 22–26
10 Mullet, J.E. and Arntzen, C.J. (1981) Biochim. Biophys. Acta 635, 236–248
11 Oettmeir, W., Masson, K. and Johanningmeer, U. (1980) FEBS Lett. 118, 267–270
12 Trebst, A. (1979) Z. Naturforsch. 34C, 986–991
13 Lien, S., McBride, J.C., McBride, A.C., Togasaki, A.K. and San Pietro, A. (1977) Photosynthetic Organelles, Special Issue of Plant Cell Physiol., 243–256
14 Arntzen, C.J., Ditto, C.L. and Brewer, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 278–282
15 Radosevich, S.R., Steinback, K.E. and Arntzen, C.J. (1979) Weed Sci. 27, 216–218
16 Renger, G. (1979) Z. Naturforsch. 34C, 1010–1014
17 Senger, H. (1977) Photosynthetic Organelles, Special Issue of Plant Cell Physiol., 229–238
18 Calveyrac, R., Dedoigt, G. and Laval-Martin, D. (1979) Planta 145, 259–267
19 Regitz, G. and Ohad, I. (1974) in Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., ed.), pp. 1615–1625, Elsevier, Amsterdam, The Netherlands
20 Regitz, G. and Ohad, I. (1976) J. Biol. Chem. 251, 247–252
21 Mattoo, A.K., Pick, U., Hoffman-Folk, H. and Edelman, M. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1572–1576
22 Ramona, A., Nelson, N. and Racker, E. (1980) Plant Physiol. 65, 730–734
23 Béliveau, R. and Bellemare, G. (1979) Biochem. Biophys. Res. Commun. 88, 797–803
24 Bennet, J., Steinback, K.E. and Arntzen, C.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5253–5257
25 Steinback, K.E., Mullet, J.E. and Arntzen, C.J. (1979) Plant Physiol. 63, Abstr. No. 147, p. 27.
26 Bennett, J. (1977) Nature 269, 344–346
27 Owens, G.C. and Ohad, I. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), vol. 3, pp. 623–630, Balaban International Science Services, Philadelphia
28 Owens, G.C. and Ohad, I. (1982) J. Cell Biol., in the press
29 Chua, N.H. and Bennoun, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2175–2179
30 Kretzer, F., Ohad, I. and Bennoun, P. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, T., Neupert, W., Sebald, W. and Werner, S., eds.), pp. 25–32, Elsevier/North-Holland, Amsterdam
31 Shochat, S., Owens, G.C., Hubert, P. and Ohad, I. (1981) Israel J. Med. Sci., Abstr.
32 Ohad, I. (1975) in Membrane Biogenesis (Tzagoloff, A., ed.), pp. 279–350, Plenum Press, New York
33 Cahen, D., Makin, S., Shochat, S. and Ohad, I. (1976) Plant Physiol. 58, 257–267
34 Bar-Nun, S., Schantz, R. and Ohad, I. (1977) Biochim. Biophys. Acta 459, 451–467
35 Gershoni, J.M. and Ohad, I. (1980) in Membrane Bioenergetics (Lee, C.P., Schatz, G. and Ernster, L., eds.), pp. 167–176, Addison-Wesley, London
36 Cahen, D., Malkin, S. and Ohad, I. (1977) Plant Physiol. 60, 845–849
37 Ohad, I., Siekevitz, P. and Palade, G.E. (1967) J. Cell Biol. 35, 553–584
38 De Petrocellis, B., Siekevitz, P. and Palade, G.E. (1979) J. Cell Biol. 44, 618–634
39 Eytan, G., Jennings, R.C., Forti, G. and Ohad, I. (1974) J. Biol. Chem. 249, 738–744
40 Gershoni, J.M. and Ohad, I. (1980) Anal. Biochem. 104, 315–320
41 Chua, N.-H. (1980) Methods Enzymol. 69, 434–446
42 Burke, J.J., Steinback, E., Ohad, I. and Arntzen, C.J. (1978) in International Symposium on Chloroplast Development (Akoyunoglou, G. and Argyroudi-Akoyunoglou, J.H., eds.), pp. 413–418, Elsevier/North-Holland, Amsterdam
43 Gershoni, J.M. and Ohad, I. (1980) J. Cell Biol. 87, 124–131
44 La Goutte, B. and Duranton, J. (1976) Biochim. Biophys. Acta 427, 141–152
45 Mullet, J., Burke, J.J. and Arntzen, C.J. (1980) Plant Physiol. 65, 814–822