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Interaction of a Phenolic Inhibitor with Photosystem II Particles

Udo Johanningmeier,[1,2] Eva Neumann,[1] and Walter Oettmeier[1]

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Abstract

Photosystem II particles have been prepared from spinach and *Chlamydomonas reinhardii* CW15 thylakoids. Photosynthetic electron transport in these particles is inhibited by phenolic compounds like dinoseb, but not by atrazine and diuron. The labeling patterns obtained by photoaffinity labels derived from either atrazine (azido-atrazine) or the phenolic herbicide dinoseb (azido-dinoseb) were compared in photosystem II particles and thylakoids. Whereas azido-atrazine in thylakoids of spinach as well as of *Chlamydomonas* labels a 32-kilodalton peptide, this label does not react in photosystem II particle preparations. Azido-dinoseb, however, labels both the thylakoid membranes and the particles, predominantly polypeptides in the 40–53 kilodalton molecular weight region. Since the latter polypeptides are probably part of the reaction center of photosystem II, it is suggested that phenolic compounds have their inhibition site within the reaction center complex. This indicates that the atrazine-binding 32-kilodalton peptide is either absent or functionally inactive in photosystem II particles, whereas the phenol inhibitor-binding peptides are not.

Key Words: Photosystem II particles; photoaffinity labels; "DCMU-type" inhibitors; phenolic-type inhibitors; photosystem II reaction center polypeptides.

Introduction

Inhibitors of photosynthetic electron transport are valuable tools for the investigation of partial reactions in the electron flow system (Trebst, 1980). In the case of PS II inhibitors two chemical classes—"DCMU-type" and phenolic inhibitors—can be distinguished (Trebst and Draber, 1979). Recent-

[1]Department of Biology, Ruhr University, D-4630 Bochum 1, FRG.
[2]Address Correspondence to: Dr. Udo Johanningmeier, Biochemie der Pflanzen, Ruhr-Universität Bochum, Postfach 10 21 48, D-4630 Bochum 1, West Germany.

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ly, radioactively labeled inhibitors of both classes which block electron flow at the reducing side of PS II were modified in such a way that they could bind irreversibly to their membrane receptor. This so-called photoaffinity labeling leads to covalent incorporation of radioactivity into the receptor protein.

Two photoaffinity labels which act as inhibitors of photosynthetic electron flow are currently available. Both are assumed to block electron transport between the primary PS II acceptor Q and the secondary acceptor, called either B or R (Bouges-Bocquet, 1973; Velthuys and Amesz, 1974). Pfister *et al.* (1981) and Gardner (1981) have shown that the herbicide atrazine[3] or its photoaffinity derivative, azido-atrazine, binds to a peptide with an apparent molecular weight of 32 kilodaltons. This is in full agreement with the earlier observations that trypsin-treated PS II particles lose DCMU sensitivity and a 32-kilodalton component simultaneously (Croze *et al.*, 1979; Mattoo *et al.*, 1981). Oettmeier *et al.* (1980) have synthesized the photoaffinity label azido-dinoseb, a derivative of the well-known herbicide dinoseb. A peptide in the 42-kilodalton region was preferentially labeled by this inhibitor. These photoaffinity labels belong to two different classes of inhibitors. Azido-atrazine is a triazine derivative and belongs to the so-called "DCMU-type" inhibitors, which all share a common structural element. Azido-dinoseb belongs to the group of phenolic inhibitors (Trebst and Draber, 1979). Although "DCMU-type" and phenol-type inhibitors block the same reaction (van Rensen *et al.*, 1978) and displace each other from the membrane, they behave differently in a number of biochemical respects (Oettmeier *et al.*, 1982a).

In several reports one to three polypeptides in the 40–53-kilodalton region have been regarded as reaction center(s) of PS II (Satoh, 1981; Satoh and Mathis, 1981; Diner and Wollman, 1980; Machold *et al.*, 1979; Chua and Bennoun, 1975; Delepelaire and Chua, 1979; Koenig and Vernon, 1981). The 42-kilodalton component labeled with the phenolic inhibitor azido-dinoseb belongs to the reaction-center candidates. In order to obtain more information on PS II polypeptides and their relation to inhibitor-sensitive electron transport, active PS II particles enriched in the reaction center were isolated and incubated with both photoaffinity labels. The results suggest the presence of a binding site of the phenolic inhibitor azido-dinoseb in the system II reaction centers.

[3]Abbreviations used: Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; azido-atrazine, 2-azido-4-ethylamino-6-isopropylamino-1,3,5-triazine; azido-dinoseb, 4-nitro-2-azido-6-isobutylphenol; BSA, bovine serum albumin; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,4-dichlorophenolindophenol; DNSJ, 2-iodo-4-nitro-6-isobutylphenol; *i*-dinoseb, 2,4-dinitro-6-isobutylphenol; DPC, 1,5-diphenylcarbazide; PS II, photosystem II.

Materials and Methods

Source of Chemicals

The photoaffinity label 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutylphenol (azido-dinoseb) was synthesized as described recently (Oettmeier *et al.*, 1980). 2-azido-4-[1-^{14}C]ethylamino-6-isopropylamino-1,3,5-triazine (azido-atrazine) with a specific activity of 19 mCi/mmol was obtained by reaction of [1-^{14}C]ethylatrazine (Amersham Buchler, Braunschweig, FRG) with trimethylamine and Na azide according to Berrer and Vogel (1970). Trypsin from bovine pancreas and trypsin inhibitor from hen egg white were purchased from Boehringer (Mannheim, FRG).

Biochemical Methods

Spinach thylakoids were prepared from spinach leaves obtained from a local market. The washed leaves were homogenized in 0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Na ascorbate, and 2 mg/ml BSA. After centrifugation at 3000 *g* for 10 min the pellet was resuspended in 5 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, and centrifuged at 21.000 *g* for 5 min. This step was repeated once.

For measuring photosynthetic activity the thylakoids were resuspended in 0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 5 mM $MgCl_2$.

Thylakoids for the PS II preparation were treated with 2 M NaBr according to Kamienietzky and Nelson (1975) for removal of the coupling factor. The pellet obtained after the salt treatment was washed once in order to remove residual NaBr.

Chlamydomonas reinhardii CW15 was grown in a medium described by Amrhein and Filner (1973). The cells were harvested in the late log phase.

For thylakoid isolation the cells were adapted to 0.4 M sucrose, dissolved in isolation medium containing 50 mM HEPES-NaOH, pH 7.5, 2 mM EDTA, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, 10 mM NaCl, and 1 mg/ml BSA. After 10-min incubation the cells were pelleted at 2000 *g* and osmotically shocked for 5 min in 5 mM HEPES-NaOH, pH 7.5, 2 mM $MgCl_2$ and 6 mg/ml BSA. The thylakoids remained in the supernatant after 3-min centrifugation at 330 *g* and could be sedimented at 4500 *g* for 5 min. Thylakoid membranes were resuspended in the isolation medium described above, giving a chlorophyll concentration of 1 mg/ml. Chlorophyll concentrations were determined by the method of Arnon (1949).

For the PS II isolation from NaBr-treated spinach thylakoids, we followed mainly the procedure as described by Satoh and Butler (1978). The

steps for PS II isolation included digitonin treatment, sucrose density gradient centrifugation, and cellulose column chromatography. The isoelectrofocusing step was omitted. PS II particles from *Chlamydomonas reinhardii* CW15 were isolated according to Diner and Wollman (1980).

Photochemical activity of thylakoids and PS II particles from spinach and *Chlamydomonas* was measured either with water (thylakoids) or DPC (particles) as electron donor. The reduction of the acceptor DCPIP was measured at 600 nm in a Zeiss PMQ II spectrophotometer modified for side illumination with white light (0.2 W/cm^2). The assay medium contained, in 1 ml, 5–10 μg Chl, 30 mM HEPES-NaOH, pH 7.0, 30 μM DCPIP, 3 mM $MgCl_2$, and, where indicated, 1 mM DPC.

Radioactive labeling with 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutylphenol (azido-dinoseb) was performed as described by Oettmeier *et al.* (1980). Labeling with azido-atrazine was done in the same way, but UV light from a mercury lamp was used instead.

Polyacrylamide gel electrophoresis (PAGE) on 11–15% acrylamide gels (30 × 19 × 0.2 cm) and sample preparation were carried out according to Chua (1980). The gels and buffers contained Li-dodecyl sulfate (LiDS) instead of the sodium salt. Samples for electrophoresis were kept at 4°C. Marker proteins were heated to 100°C for 5 min. Two sets of markers were used: (1) a mixture of phosphorylase B (94 kilodaltons), BSA (67 kilodaltons), ovalbumin (43 kilodaltons), carbonic anhydrase (30 kilodaltons), soybean trypsin inhibitor (20.1 kilodaltons), and α-lactalbumin (14.4 kilodaltons) from Pharmacia (Uppsala, Sweden), or (2) phosphorylase B (92.5 kilodaltons), BSA (66.2 kilodaltons), ovalbumin (45 kilodaltons), and lysozyme (14.4 kilodaltons) from Bio-Rad (Richmond, California). Gels were stained for proteins with Serva Blue G. Radioactivity was detected by slicing the gels as described earlier (Oettmeier *et al.*, 1980).

Results

Characterization and Influence of Different Inhibitors on the Activity of PS II Particles

Several PS II particle preparations have so far been reported. The purification steps include mechanical rupture of the thylakoid membranes, and detergent treatment followed by density centrifugation and/or column chromatography. One PS II preparation has been well characterized in terms of polypeptide composition by Satoh (1979, 1981). After a final isoelectrofocusing step, he obtained a pigment protein complex consisting of three polypeptides with apparent molecular weights of 43, 27, and 6 kilodaltons. Sometimes a fourth peptide with a molecular weight of 54 kilodaltons was also

found. This complex was photosynthetically active using the artificial electron donor DPC and the acceptor DCPIP. We have prepared PS II particles from spinach and from a cell wall-free mutant of *Chlamydomonas reinhardii*.

Our PS II preparation from spinach thylakoids was obtained following mainly Satoh's procedure with some modifications. The coupling factor was removed from the membrane by NaBr (Kamienietzky and Nelson, 1975) instead of EDTA treatment. The purification steps included digitonin extraction, isopycnic centrifugation in sucrose, and DEAE-cellulose column chromatography. PS II particles obtained from the density gradient initially showed photosynthetic activity of up to 60 μmol DCPIP/mg Chl/hr, depending on the quality of the spinach. After freezing and thawing, this activity decreased significantly to less than half of the original value.

A similar active preparation was obtained with PS II particles isolated from *C. reinhardii* CW15. They were prepared according to the method described by Diner and Wollman (1980). Thylakoids from this green alga were obtained by osmotic rupture of the cells instead of using the French press. This was possible because the CW15 mutant of *C. reinhardii* has no cell wall (Davies and Plaskitt, 1971). PS II particles from the alga thylakoids had a photosynthetic activity of 37 μmol DCPIP/mg Chl/hr.

A number of different PS II inhibitors affected the electron transport activity of these two preparations. As has been shown previously, inhibitors of the "DCMU type" and phenolic type have different binding sites and binding peptides on the reducing side of PS II (Oettmeier *et al.*, 1982a). In order to correlate the inhibitor sensitivity of PS II electron transport and polypeptide composition, we have compared the electron flow inhibition from DPC to DCPIP and from H_2O to DCPIP in PS II particles and thylakoids, respectively (Table I). As can be seen, PS II particles from spinach as well as from

Table I. Effect of Different Inhibitors on Electron Flow in Thylakoids and Photosystem II Particles[a]

	I_{50}(μM)			
	Spinach		*C. reinhardii* CW15	
	Thylakoids	PS II	Thylakoids	PS II
"DCMU type"				
DCMU	0.05	>>10	0.025	>>10
Atrazine	0.3	>>10	1.25	>>10
Phenol type				
DNSJ	0.1	0.4	0.55	3.0
i-dinoseb	1.0	5.5	5.5	8.0

[a]The I_{50} value is the concentration needed for 50% electron transport inhibition. Electron flow was measured either from H_2O (thylakoids) or from DPC (PS II particles) to DCPIP. For conditions see Materials and Methods.

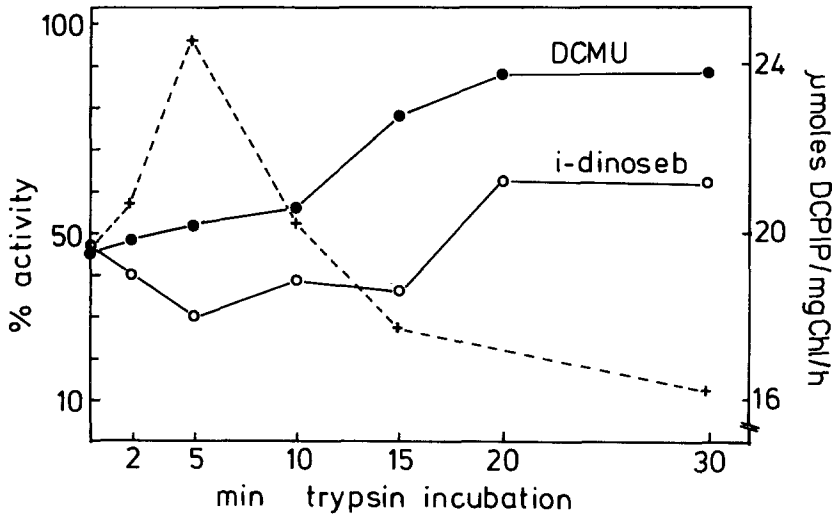
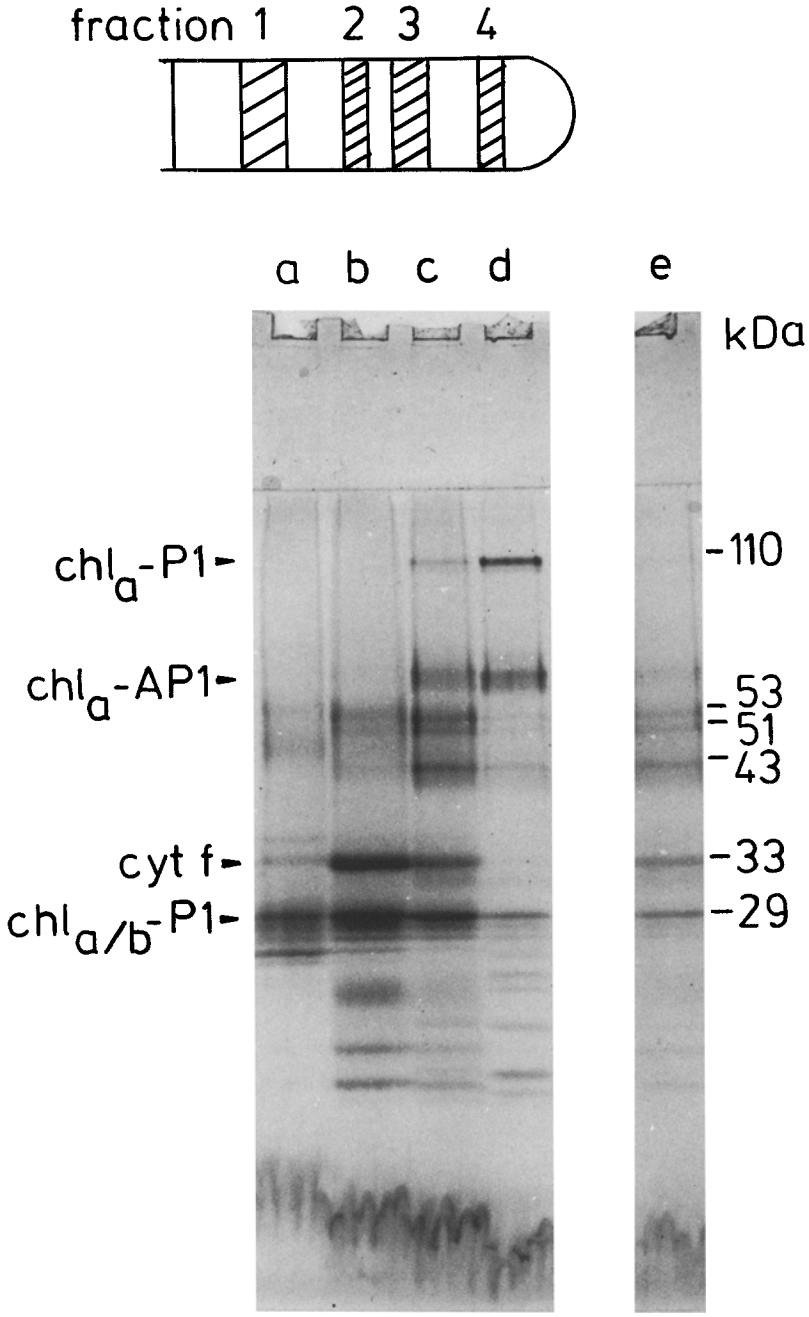


Fig. 1. Effect of trypsin on the DCMU- and *i*-dinoseb-inhibited electron transport rate in partially purified spinach PS II particles. The dotted line shows the effect of the protease on the control rate (right ordinate). The incubation medium contained in a volume of 0.6 ml: PS II particles with a chlorophyll content of 75 μg, 200 μg trypsin, 40 mM Tricine-NaOH, pH 8.0, and 4 mM $MgCl_2$. Incubation was stopped by addition of trypsin inhibitor, and a 100-μl aliquot was measured in the reaction medium (see Materials and Methods) containing (with the exception of the control) either 3 μM DCMU or 3 μM *i*-dinoseb.

the alga are essentially insensitive to the two representatives of "DCMU-type" herbicides, atrazine and DCMU. The phenol sensitivity, however, is slightly reduced, but not nearly as much as compared to "DCMU-type" inhibitors.

At an earlier purification step, we sometimes obtained PS II particles which retained an appreciable sensitivity toward DCMU (I_{50} about 3 μM). Trypsin treatment is known to overcome DCMU inhibition in thylakoid membranes (Regitz and Ohad, 1976; Renger, 1976; Tischer and Strotmann, 1979; Steinback *et al.*, 1981). The same could be demonstrated for PS II particles at an earlier purification step. In these particles trypsin treatment completely removed the inhibitor effect after a certain incubation time (Fig. 1). However, this is only valid for DCMU. The substituted phenol *i*-dinoseb

Fig. 2. Polypeptide pattern from four different bands obtained by sucrose density centrifugation of digitonin-treated spinach thylakoids (lines a–d, corresponding to fractions 1–4 in the centrifuge tube which is illustrated at the top of the gel) and from spinach PS II particles further purified by DEAE-cellulose chromatography (line e). Solubilized samples of fractions 1–4 containing 25 μg Chl were electrophoresed in slots a–d, respectively. 10 μg Chl was applied to slot e. Some thylakoid polypeptides identified earlier are indicated for reference.


fraction 1 2 3 4
a b c d
e
kDa
chl$_a$-P1
chl$_a$-AP1
cyt f
chl$_{a/b}$-P1
110
53
51
43
33
29

becomes a better inhibitor during the first 15 min of protease action, and inhibition is reduced to a certain extent only after longer treatment. It is interesting that in the first minutes of trypsin treatment the rate of the DCPIP reduction becomes accelerated (dotted line in Fig. 1, right scale). During this time the acceptor DCPIP possibly has a better accessibility to its reduction site.

Polypeptide Composition of PS II Particles Isolated from Spinach and from C. reinhardii CW15 Thylakoids

Figure 2 shows the polypeptide profiles of four different fractions from the sucrose density gradient on 11–15% polyacrylamide gels (lines a to d) derived from digitonin-treated spinach thylakoids. In addition, the polypeptide profile of purified PS II particles after DEAE-cellulose chromatography is also depicted (Fig. 2, line e). Fraction 3 from the gradient exhibited the highest photosynthetic activity (34 μmol DCPIP/mg Chl/hr) as compared with fractions 1, 2, and 4 (0, 16, and 15 μmol DCPIP/mg Chl/hr, respectively). The highest PS II activity coincides with the appearance of somewhat smeared polypeptide bands in the molecular weight range from 40–43 kilodaltons and two bands around 51 and 53 kilodaltons. These bands are the only heavily stained ones which increase from fraction 3 to 4.

Fraction 3 was further purified on a DEAE-cellulose column. This step removed mainly PS I contamination. Only traces of the P700-Chl_a-protein (Chl_a-P1) and the corresponding apoprotein (Chl_a-AP1) are present (Fig. 2) in the purified particles.

Besides the polypeptides already mentioned, two other proteins are prominent (Fig. 2, line e): a 33- and a 29-kilodalton component. The 33-kilodalton polypeptide is very likely contaminating cytochrome f. This band stains with the heme stain (Thomas *et al.*, 1976; not shown) and appears to be a major component of the reddish-brown fraction 2 from the density gradient (Fig. 2, line b). The green 29-kilodalton peptide and minor bands around 28 and 27 kilodaltons possibly belong to the light-harvesting Chl *a*/*b* protein ($Chl_{a/b}$-P1), a contaminant present in all fractions from the gradient, which could not be removed by column chromatography.

Four polypeptides with molecular weights around 21, 19.5, and 17.5 kilodaltons are hardly visible on the gel (Fig. 2, line e). These bands also appear not to be related to PS II activity since they are more prominent in other fractions obtained from the gradient. Heating the solubilized samples shown in Fig. 2 to 100°C for 5 min leads to aggregations of the 40–43-, 51-, and 53-kilodalton components. These aggregations are not separated on the gel (not shown). This same effect was described by Satoh (1981) for the reaction center polypeptides.

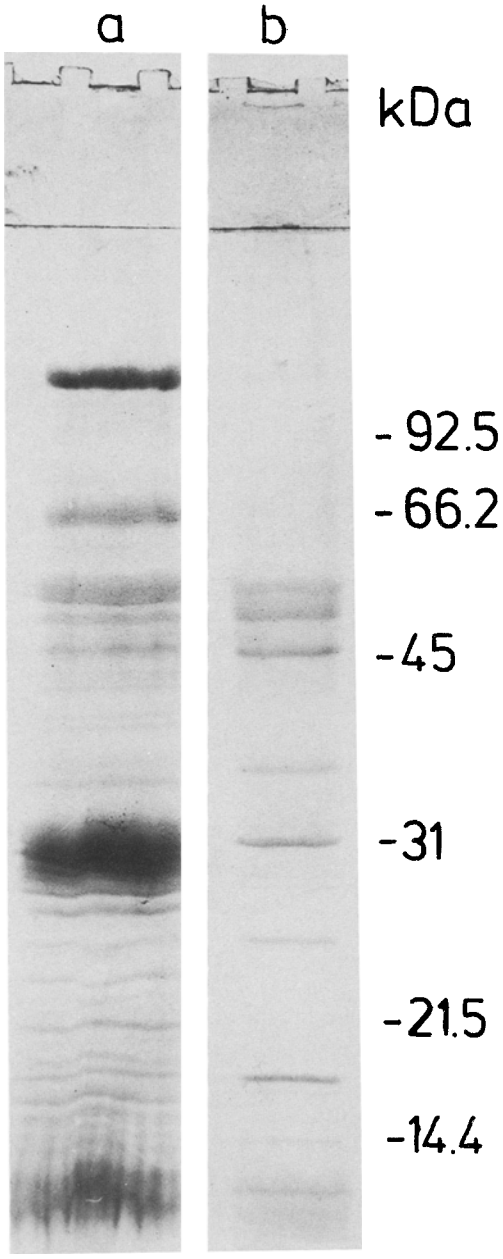


Fig. 3. Polypeptide pattern from *C. reinhardii* CW15 thylakoids (line a) and from PS II particles isolated from this alga (line b). The small arrows indicate peptides with apparent molecular weights of 47 and 51 kilodaltons. Samples containing 50 and 16 μg Chl were applied to the slots a and b, respectively.

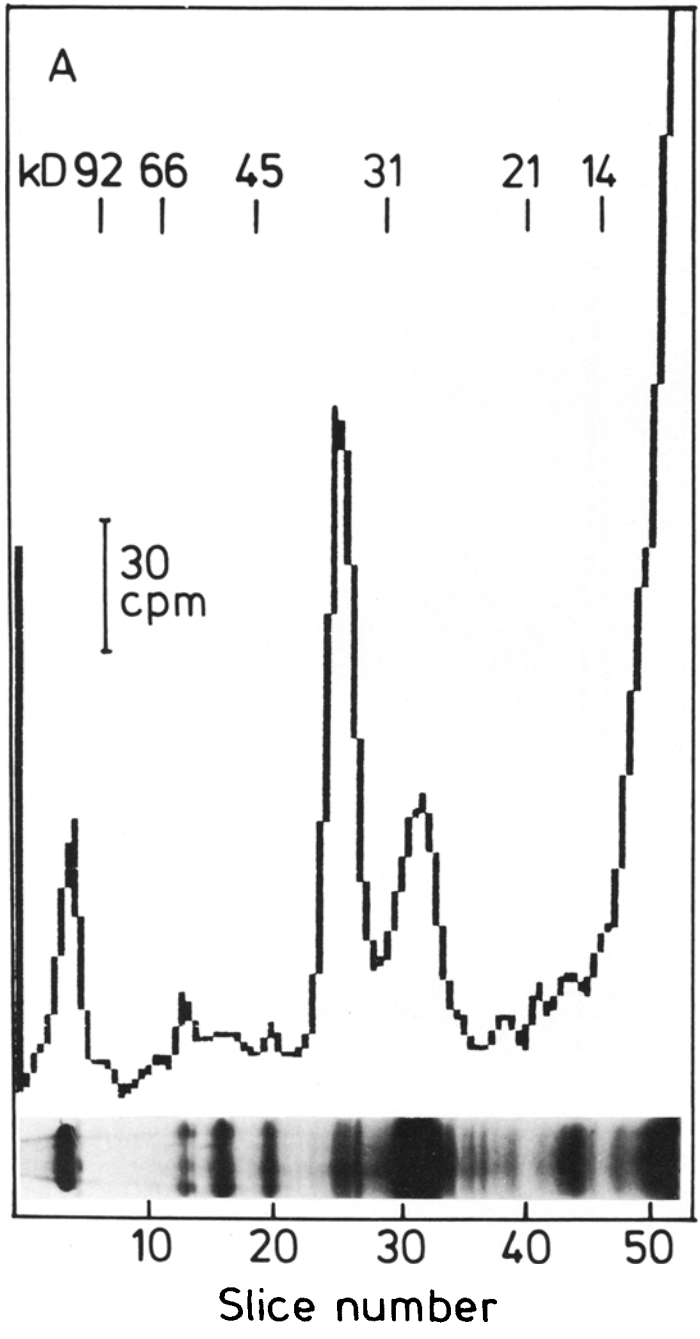
C. reinhardii CW15 PS II particles obtained from the density gradient can be separated into six to seven polypeptide bands on 11–15% polyacrylamide gels. As can be seen from Fig. 3, the most prominent bands have apparent molecular weights of 55, 51, 47, 35, 30, 24, and 17 kilodaltons. This preparation exhibits photosynthetic activity of 37 μmol DCPIP/mg Chl/hr with DPC as electron donor. In contrast to spinach PS II particles, these preparations are able to use H_2O as electron donor, although with very low rates (about 6 μmol DCPIP/mg Chl/hr). For comparison, Fig. 3 also shows the polypeptide pattern of whole *C. reinhardii* CW15 thylakoids. The 51- and 47-kilodalton polypeptides (arrows at line a, Fig. 3) aggregate upon heating, as is the case for the analogous peptides in solubilized spinach thylakoids. The 55-kilodalton component most likely belongs to the α and β subunits of the coupling factor (Chua and Blomberg, 1979). It was found that this protein was indeed present in the PS II particles from the alga since the particles precipitated in the presence of spinach CF_1 antisera (not shown).

Photoaffinity Labeling of Thylakoids and PS II Particles

Photoaffinity labeling has been a useful procedure for identification of receptor binding sites (Bayley and Knowles, 1977). For identification of herbicide binding sites in the thylakoid membrane two photoaffinity labels are presently available: azido-atrazine (Gardner, 1981) and azido-dinoseb (Oettmeier *et al.*, 1980). Labeling patterns of thylakoids in comparison to PS II particles of spinach and of *C. reinhardii* were performed with both photoaffinity labels. The results are shown in Figs. 4 and 5. Using the photoaffinity label azido-atrazine, we obtained the results of Gardner (1981) and Pfister *et al.* (1981), i.e., a 32–34-kilodalton peptide is a receptor for the "DCMU-type" inhibitor atrazine in spinach thylakoids (Fig. 4A). As can be seen from Fig. 5A, this is also true for *Chlamydomonas* thylakoids. PS II preparations from both spinach and *Chlamydomonas* failed to bind the photoaffinity label azido-atrazine. This is judged from the absence of any radioactivity in polypeptides in the 32–34-kilodalton region (Figs. 4C and 5C). The loss of sensitivity toward "DCMU-type" inhibitors in these PS II particles (Table I) is in accordance with this result.

It has previously been shown that a radioactive phenolic inhibitor with a photolabile azido group, i.e., 4-nitro-2-azido-6-[2′,3-^{3}H]isobutyl-phenol (azi-

Fig. 4. Radioactivity scans and corresponding polyacrylamide gels of thylakoid membranes (A, B) and PS II particles (C, D) from spinach labeled with ^{14}C-azido-atrazine (A, C) and ^{3}H-azido-dinoseb (B, D). Thylakoids and particles were incubated under UV light for 15 min with 17 and 500 nmol azido-atrazine/mg chl (17 and 7 μM), respectively. Thylakoids and particles labeled with azido-dinoseb were illuminated with white light for 15 min in the presence of 10 and 50 nmol azido-dinoseb/mg chl (2.5 and 2 μM), respectively. The positions of different marker proteins are indicated.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30
cpm
10 20 30 40 50
Slice number

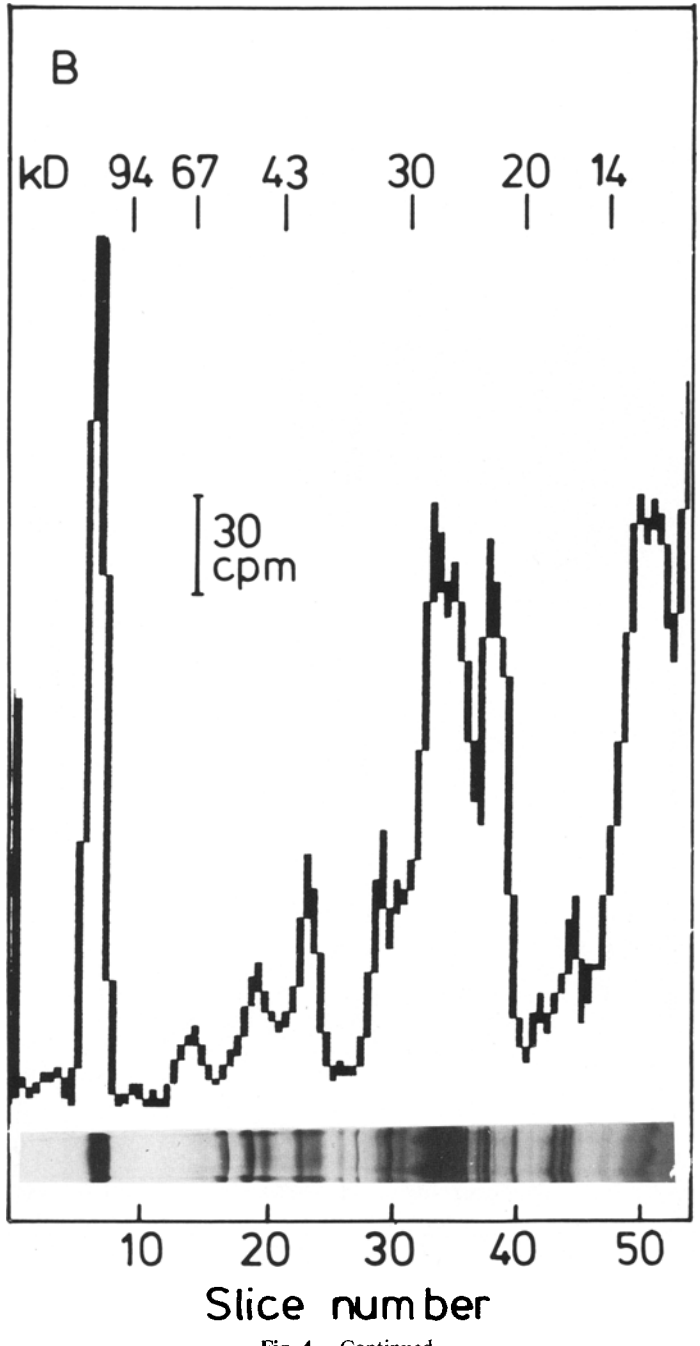
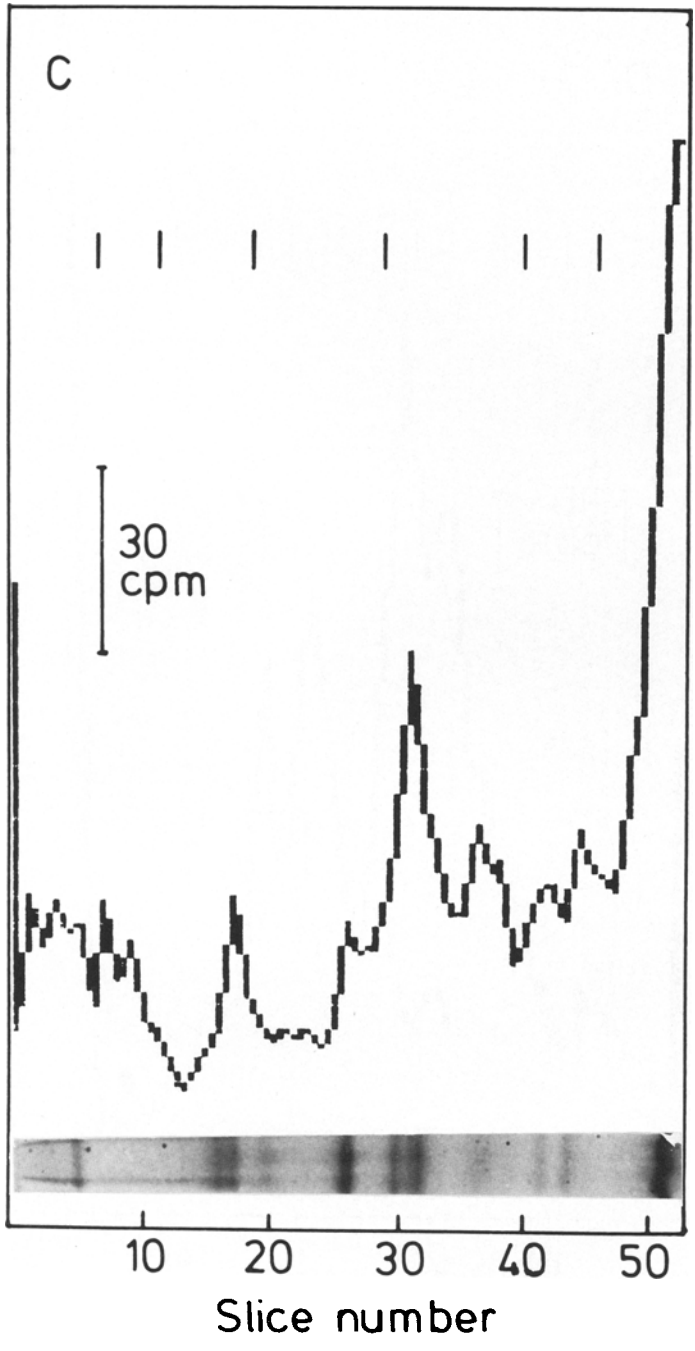


Fig. 4. Continued.



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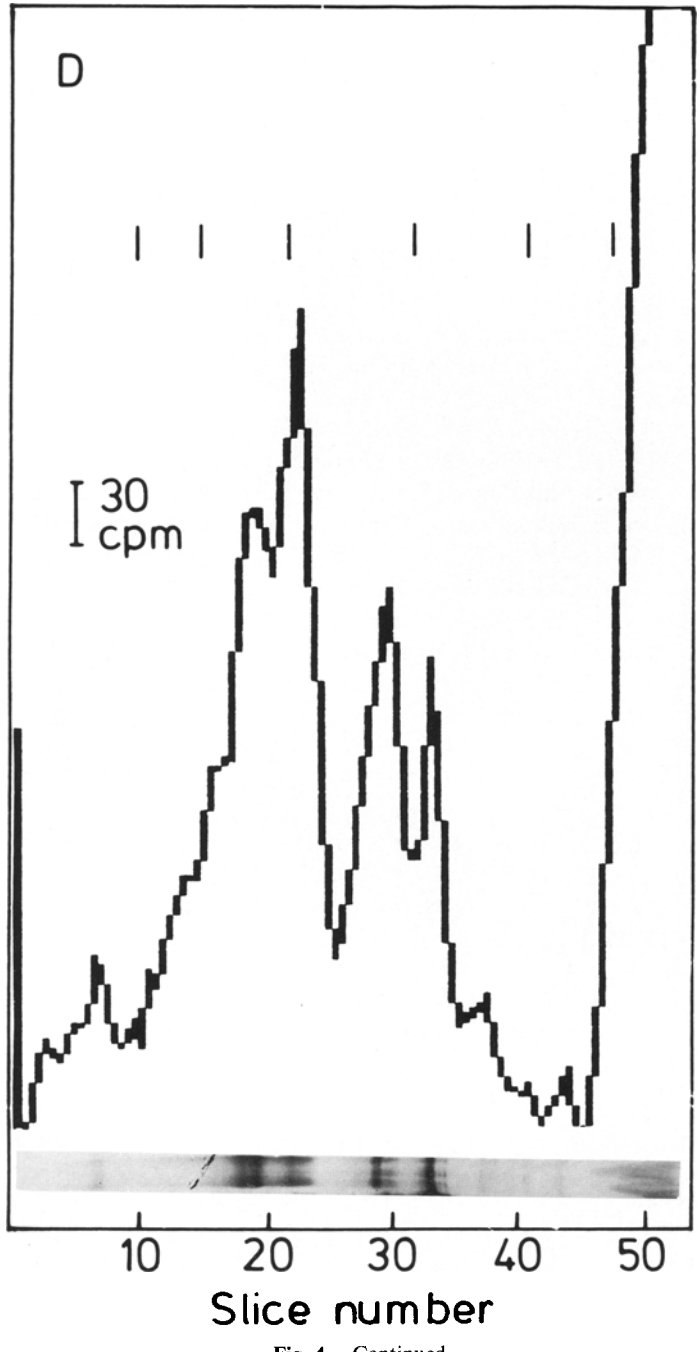


Fig. 4. Continued.

do-dinoseb), preferentially reacts in spinach thylakoids with a polypeptide in the 42-kilodalton region, and also with peptides in the 32-, 25- (LHCP), and 17-kilodalton regions (Oettmeier *et al.* 1982a). The high amount of radioactivity in the free pigment zone was attributed to unspecific binding to lipids and pigments. In the experiment shown in Fig. 4B the labeling pattern of spinach thylakoids after reaction with azido-dinoseb is somewhat different from that in the papers of Oettmeier *et al.* (1980, 1982a). The most prominent band belongs to the Chl_a-P1, a 110-kilodalton polypeptide containing P700. This was not observed earlier and is due to the relatively mild solubilization procedure (sample solubilization and electrophoresis at 4°C), which preserves a high amount of chlorophyll attached to the protein. Upon heating the sample, this radioactivity peak is diminished (not shown).

Besides the 110–kilodalton peptide, six other radioactivity peaks with molecular weights around 53, 43, 33, 28, 24, and 17 kilodaltons can be detected (Fig. 4B). Possibly also due to the different solubilization conditions, the peak around 43 kilodaltons is not very prominent. We wish to emphasize the 53-kilodalton protein peak. Although it was observed in earlier reports (see Figs. 3 and 5 in Oettmeier *et al.*, 1980 and 1982a, respectively), not much attention was paid to it. The importance of the 53-kilodalton peptide becomes obvious from the labeling experiments with PS II particles (Fig. 4D). In PS II particles only two peaks are prominent, the 43- and the 51–53-kilodalton peptides. The diffuseness of this peak coincides with the smeared zone in the Coomassie blue-stained gel. A similar situation is observed comparing azido-phenol-labeled thylakoids from *C. reinhardii* CW15 and PS II particles from this alga (Fig. 5B and D). In the thylakoid preparation the chlorophyll-containing 110-kilodalton polypeptide (CP I) and the apoprotein of CP I with a molecular weight around 66 kilodaltons (polypeptide 2; Girard *et al.*, 1980) are labeled. Furthermore, radioactivity can be found in the 40–47- and the 23–30-kilodalton region, in a 17-kilodalton polypeptide, and a protein below 14 kilodaltons. As with spinach, *Chlamydomonas* PS II particles show most of the radioactivity in the 43–53-kilodalton region, reflecting the smeared zone on the stained gel.

Discussion

The reducing side of PS II has recently gained special interest. At this site not only are electrons and protons transported to the plastoquinone pool, but herbicides also act here and block electron flow between Q and B (Trebst *et al.*, 1979; van Rensen *et al.*, 1978). These inhibitors can be arranged into two chemical classes, the "DCMU-type" and phenolic inhibitors (Trebst and Draber, 1979). It became evident that the site of inhibitor action is a protein

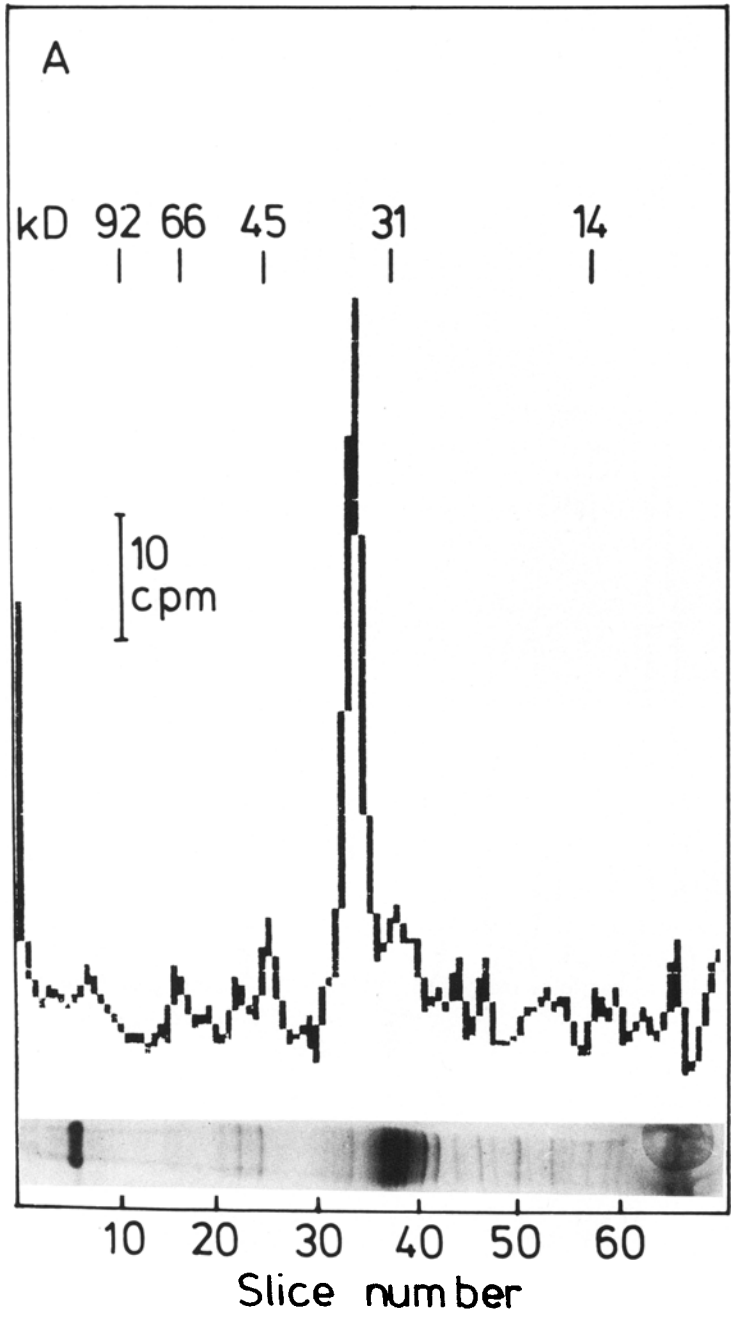
because of its protease lability, i.e., sensitivity to certain inhibitors is lost upon trypsin treatment (Regitz and Ohad, 1976; Renger, 1976). It is now common to involve a B-protein on the acceptor side of PS II, participating in electron flow from the primary acceptor Q to the main plastoquinone pool. The first indication for a 32-kilodalton component being a subunit of this membrane protein was reported by Croze *et al.* (1979). This was confirmed by others and finally directly shown with a photoaffinity label, which preferentially reacts with a 32-kilodalton polypeptide (Pfister *et al.*, 1981; Gardner, 1981; Mullet and Arntzen, 1981). The labeling experiment was done with azido-atrazine, an inhibitor of the "DCMU-type." Experiments with a photoaffinity label belonging to the other inhibitor class of the substituted phenols revealed, however, that another polypeptide in the 42-kilodalton region was labeled preferentially (Oettmeier *et al.*, 1980). This then seems to indicate that the B-protein is composed of two subunits: a 32- and a 42-kilodalton peptide.

Indeed, "DCMU-type" and phenolic inhibitors at first appeared to have an identical mode of action (Reimer *et al.*, 1979; van Rensen *et al.*, 1978), but later differences between the two classes became apparent. Some of the differences are: (i) differential effects upon very short trypsin treatment (Steinback *et al.*, 1981; Böger and Kunert, 1979); (ii) increased sensitivity of thylakoids from atrazine-resistant Amaranthus mutants to phenolic inhibitors (Pfister and Arntzen, 1979; Oettmeier *et al.*, 1982b); (iii) the time lag observed by inhibition with phenols (Reimer *et al.*, 1979), which is overcome after a short trypsin treatment (Böger and Kunert, 1979); (iv) phenol inhibitor-sensitive but atrazine-insensitive electron flow in Triton PS II particles (Mullet and Arntzen, 1981); and (v) the fact that the quantitative correlation of inhibitory potency to chemical structure follows different equations with quite different substitution parameters (Trebst and Draber, 1979). As already mentioned, different polypeptides are labeled with azido-atrazine as compared with azido-dinoseb.

The experimental results presented here support the concept that the sites of inhibition and binding of the two inhibitor groups are not identical.

1. PS II particles from both spinach and *Chlamydomonas reinhardii* CW15 thylakoids catalyze electron flow from DPC to DCPIP. This reaction is sensitive to phenol-type inhibitors like *i*-dinoseb and DNSJ; in contrast, it is

Fig. 5. Radioactivity scans and corresponding polyacrylamide gels of thylakoid membranes (A, B) and PS II particles (C, D) from *C. reinhardii* CW15 labeled with ^{14}C-azido-atrazine (A, C) and ^{3}H-azido-dinoseb (B, D). Thylakoids and particles labeled with azido-atrazine were incubated under conditions given in Fig. 5 with 15 and 100 nmol azido-atrazine/mg chl (15 and 4 μM), respectively. Thylakoids and particles labeled with azido-dinoseb were incubated under the same conditions given in Fig. 5 with 5 and 50 nmol azido-dinoseb/mg chl (1 and 2 μM), respectively. The positions of different marker proteins are indicated.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kD 92 66 45 31 14
10
cpm
10 20 30 40 50 60
Slice number

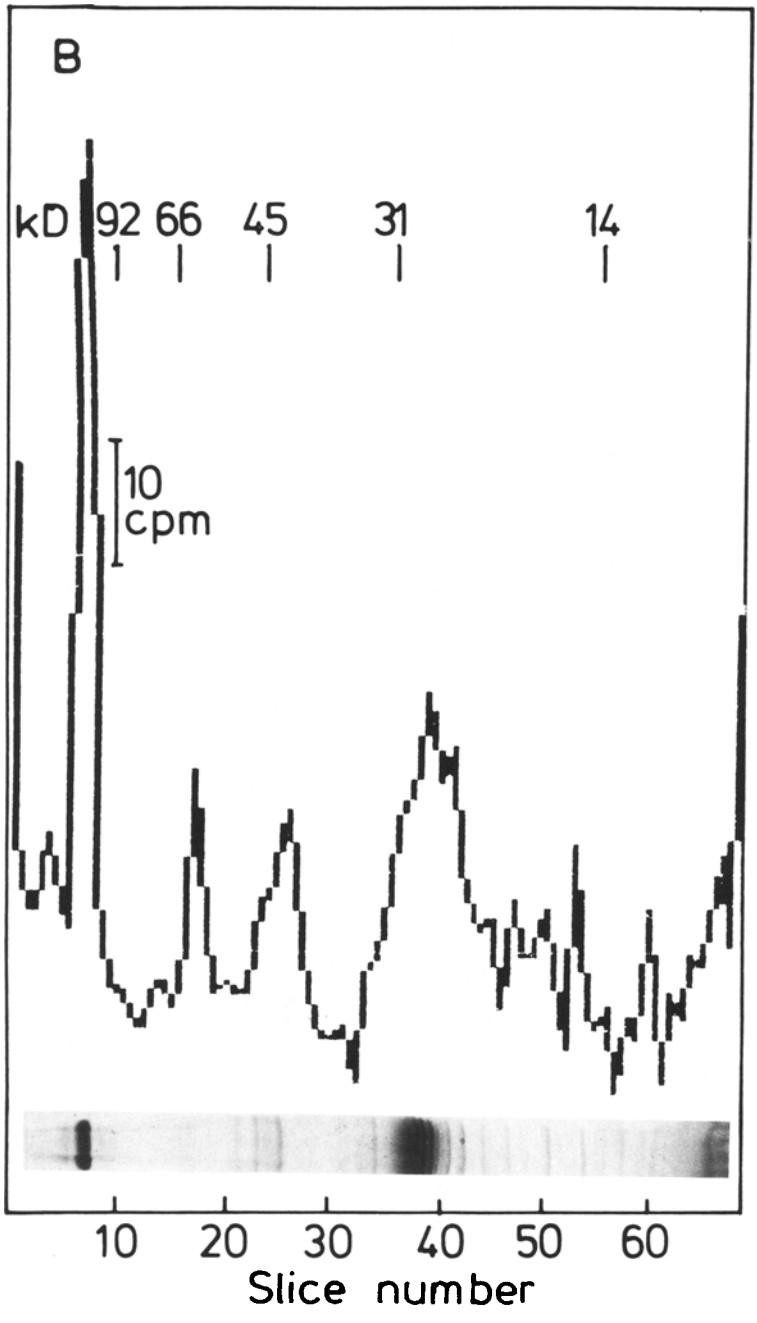

Fig. 5. Continued.

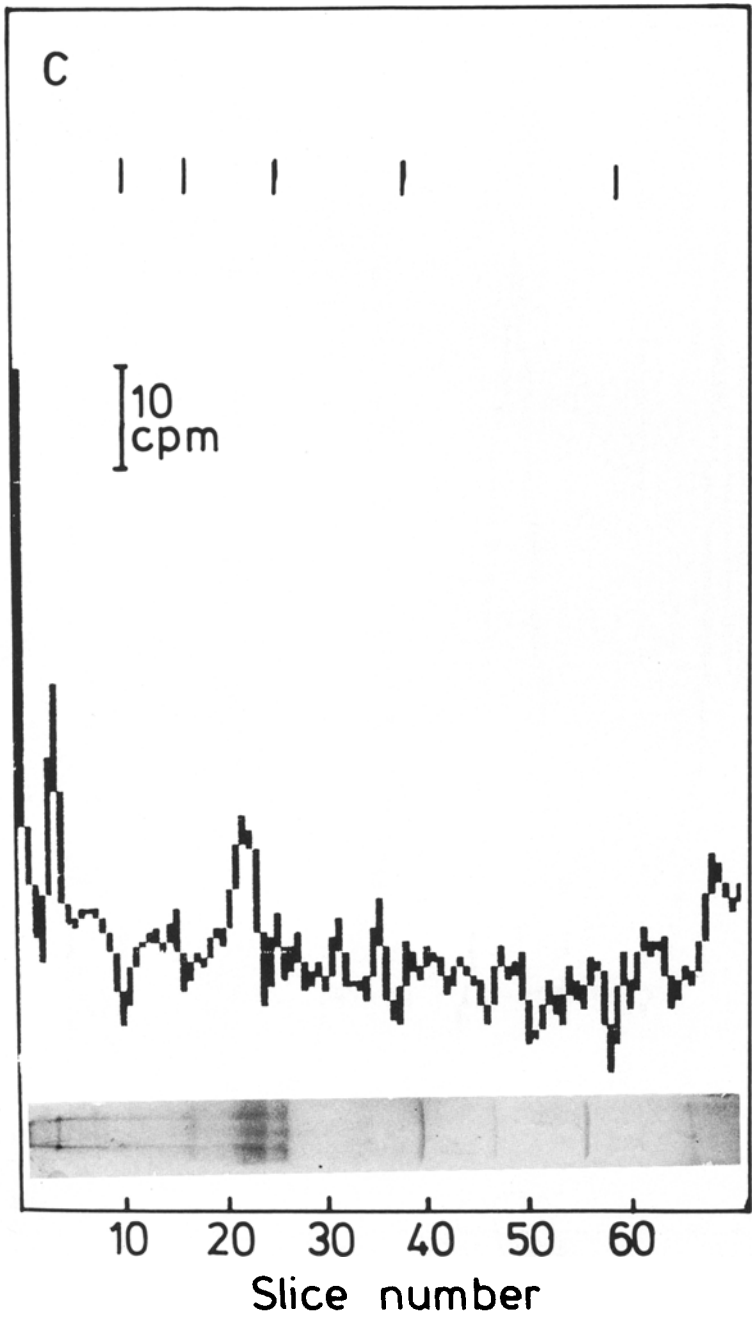

Fig. 5. Continued.

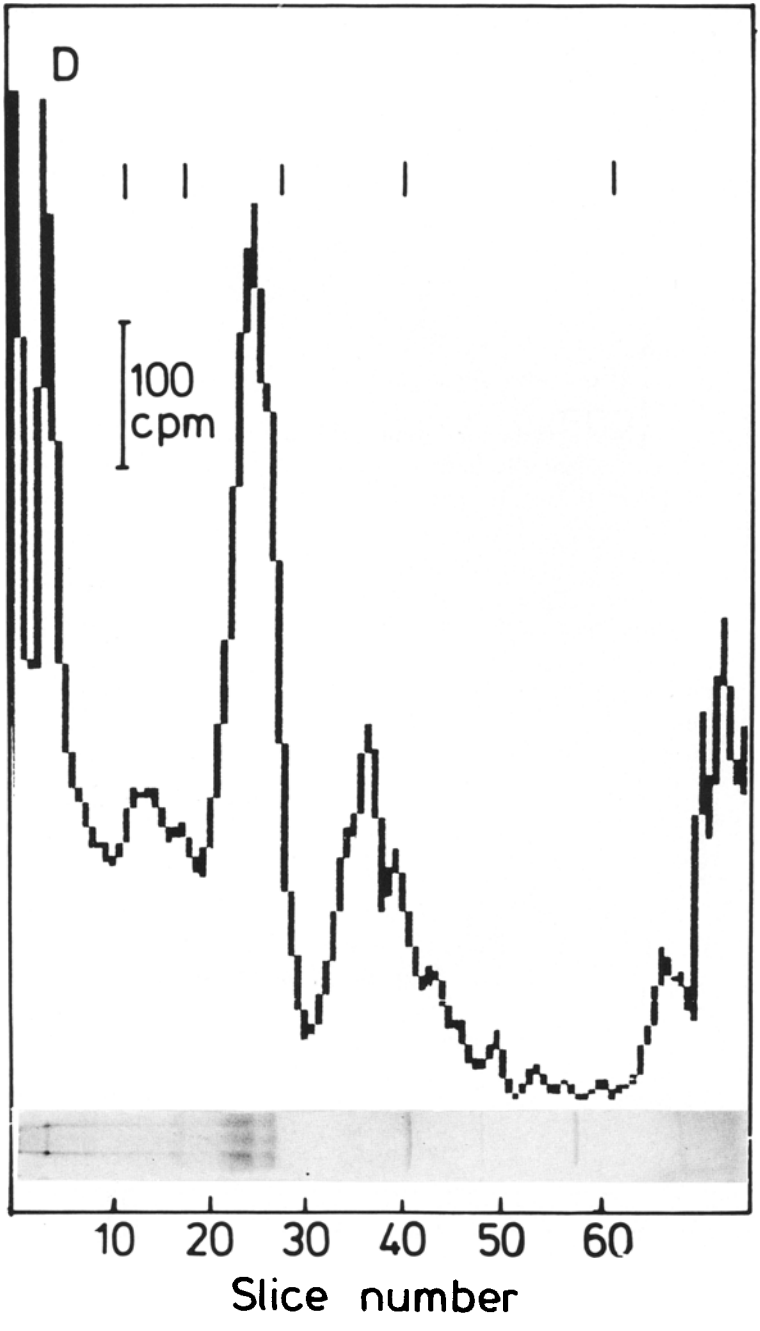

Fig. 5. Continued.

not sensitive toward DCMU or atrazine. In intact thylakoid membranes electron flow is sensitive to both types of inhibitors.

2. The two photoaffinity labels azido-atrazine and azido-dinoseb label the intact thylakoids from both spinach and *Chlamydomonas* on a 32-kilodalton and, among others, a 42- and 53-kilodalton peptide, respectively. In PS II particles from both organisms azido-atrazine no longer reacts, whereas azido-dinoseb shows labeling in the 40–53-kilodalton region.

3. The phenol-binding 40–53-kilodalton peptides copurify during the isolation of the reaction center complex. This is in accordance with the finding that the phenols DNSJ and *i*-dinoseb still inhibit electron flow in PS II particles at very low concentrations.

4. Upon trypsin treatment the sensitivity of PS II particles toward phenol inhibitors in the first minutes slightly increases and is lost to a large extent after prolonged incubation.

Although different, the inhibition and binding sites for the two inhibitor classes have to be spatially close to each other. This was concluded from displacement experiments, in which one class of inhibitors can be replaced by inhibitors from the other (Reimer *et al.*, 1979; Oettmeier and Masson, 1980). Short trypsin treatment of PS II particles, which still have retained some DCMU sensitivity (and thus should contain at least partially intact 32-kilodalton peptides), decreases the *i*-dinoseb-inhibited electron transport rate as compared to the undigested control (Fig. 1). This protease treatment could well remove the barrier consisting of the 32-kilodalton peptide and allow better access of the phenolic inhibitor to its inhibition site.

The results presented here furthermore indicate that the peptides carrying the phenol inhibitor binding sites are more fundamental for the functioning of PS II than the 32-kilodalton peptide. This peptide can be removed (or functionally dissociated or inactivated) from the PS II particles. The phenol binding peptides are probably more buried within the membrane than the 32-kilodalton peptide. Our trypsin-treatment experiments and those of others (Böger and Kunert, 1979; Steinback *et al.*, 1981) may even be interpreted to indicate that the 32-kilodalton peptide is on top of the phenol binding peptides.

We suggest that the phenol inhibitor binding peptides belong to the reaction center complex of PS II. Although the polypeptide composition of the core complex is still a matter of debate, there are up to three peptides in the 40–53-kilodalton region involved in the arrangement of the complex (Delepelaire and Chua, 1979; Chua and Bennoun, 1975; Machold *et al.*, 1979; Diner and Wollman, 1980; Satoh, 1981; Satoh and Mathis, 1981). By comparison of the polypeptide composition of the different fractions from the density gradient with the PS II activity in these fractions (Fig. 2), it can be seen that

with the appearance of three polypeptides with molecular weights of 43, 51, and 53 kilodaltons the highest electron transport activity can be measured. These peptides also preferentially bind azido-dinoseb.

The scheme in Fig. 6 indicates this in a simplified way. It raises the question whether the plastoquinone binding site on the B-protein is on the 32-kilodalton or the phenol-binding peptides. The loss of activity of artificial electron acceptors of PS II in intact thylakoids (which presumably compete with plastoquinone) after trypsin treatment would suggest that the 32-kilodalton peptide carries the plastoquinone binding site.

However, there exists some doubt whether the 32-kilodalton peptide is essential for a functioning electron transport system. In PS II particles DCPIP reduction at the expense of DPC is possible. In trypsinized thylakoids DCPIP

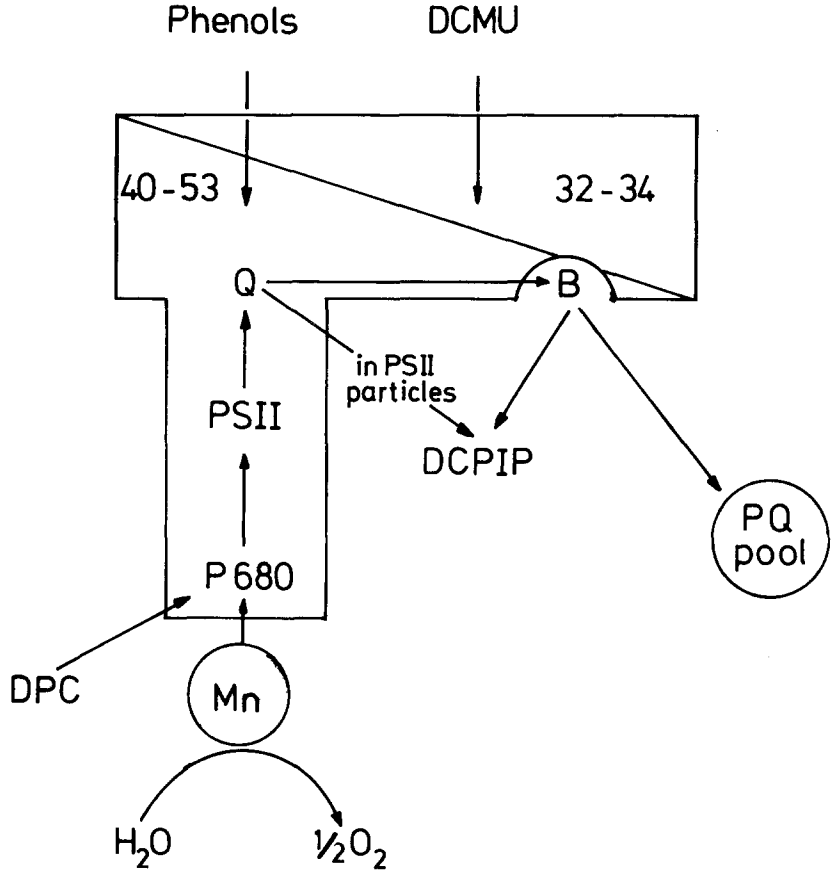

Fig. 6. Scheme of the PS II complex with inhibitor binding proteins represented by 32–34 and 40–53 kilodaltons as their approximate molecular weight regions.

reduction has been reported by Regitz and Ohad (1976) and by Tischer and Strotmann (1979), in contrast to Jansson *et al.* (1979). Both PS II particles and trypsinized thylakoids should not contain the intact 32-kilodalton peptide. More recently it has been demonstrated that mutants of algae and plants which are depleted of the 32-kilodalton peptide nevertheless retain an active photosynthetic electron transport system (Astier and Joset-Espardellier, 1981; Gressel, 1982). Concerning the peptides in the 40–53-kilodalton region, it is very likely that they are obligatory components of electron flow through PS II.

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