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Probing for the interaction of the 32 kDa-Q_B protein with its environment by use of bifunctional cross-linking reagents

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Chlamydomonas reinhardtii **thylakoid polypeptides were cross-linked with hydrophobic or hydrophilic homo- or hetero-bifunctional reagents, including cleavable cross-linkers containing a dithiol group (3,3-dithiobis(succinimidylpropionate), *N*-succinimidyl-3-(2-pyridyldithio)propionate and 3,3-dithiobis(sulfosuccinimidylpropionate), abbreviated to DTSP, SPDP and DTSSP, respectively) or glutaraldehyde. None of these reagents cross-linked the 32 kDa-Q_B-protein, as identified by specific radioactive labeling and reaction with antibodies. Treatment of thylakoids with β-D-octylglucoside prior to addition of all the cross-linkers used, resulted in effective cross-linking of the 32 kDa-Q_B-protein. Treatment of thylakoids with cross-linkers, followed by removal of the non-reacted reagents and addition of β-D-octylglucoside, caused extensive cross-linking of the 32 kDa-Q_B-protein by SPDP and, to a lesser extent, by DTSP, indicating a possible formation of monovalent links with the 32 kDa-Q_B-protein in the native membrane. The 32 kDa-Q_B-protein was not cleaved by trypsin in thylakoids treated with DTSP, DTSSP or glutaraldehyde, while SPDP did not have a similar protective effect. Photosystem II activity and the affinity for Diuron binding and/or the number of binding sites were also affected by cross-linkers. None of the cross-linkers prevented the light-induced degradation of the 32 kDa-Q_B-protein in isolated thylakoids exposed to high light intensity. It is suggested that the 32 kDa-Q_B-protein is confined within a specific hydrophobic environment which might be essential for its function and prevent the cross-linking of the 32 kDa-Q_B-proteins –SH or $-HN_2$ groups with similar reactive groups of neighboring proteins. Alteration of the membrane lipid phase by detergent treatment allows cross-linking to occur.**

Introduction

The 32 kDa thylakoid protein which participates in the formation of the Q_B secondary quinone acceptor of Photosystem II and the binding site for herbicides of the urea and triazine family turns over in vivo at rates exceeding by far the turnover of all other thylakoid polypeptides [1,2]. The turnover of this protein is light dependent and proportional to the light intensity [3]. When the rate of degradation is faster than that of its synthesis, the thylakoids might become depleted of the 32 kDa protein, and electron flow via Photosystem II is inhibited. This phenomenon was described to occur both in higher plants [1] and algae [2], and is thought to be the major cause of photoinhibition [4,5]. We have recently been able to show that the light-dependent specific degrada-

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Abbreviations: DCIP, dichlorophenolindophenol; DCMU, (Diuron) 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMSO, dimethylsulfoxide; DTSP, 3,3-dithiobis(succinimidylpropionate); DTSSP, 3,3-dithiobis(sulfosuccinimidylpropionate); SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate.

tion of the 32 kDa protein occurs also in isolated thylakoids in vitro in the absence of chloroplast-soluble proteins [6,7], and thus all components responsible for this process reside within the thylakoid membrane.

It has been reported before that the 32 kDa polypeptide is present in Photosystem II (PS II) preparations isolated from higher plants [8] and algae [9,10]. Thus, one would expect it to be localized primarily in the grana region of the chloroplast thylakoids [11]. Since the light-dependent turnover of the 32 kDa polypeptide appears to involve the participation of a membrane-bound protease(s) activity [6], it would be expected that this enzyme will also be a part of the PS II complex. Most of the polypeptides participating in the formation of a functional PS II complex have already been identified in chloroplasts from various sources, including higher plants [9] and unicellular algae [10]. Based on these data, specific functions could be ascribed to almost all polypeptides present in isolated PS II complexes, so that none of these identified polypeptides could account for a putative protease. It is quite possible that additional polypeptides which do not participate directly in the process of PS II electron flow but might be involved in maintaining its structural integrity or assembly and, eventually, in the turnover process of the 32 kDa polypeptide, might exist but are lost during the process of isolation of the PS II complex.

In this work we have attempted to identify such polypeptides located in close proximity to the 32 kDa herbicide-binding protein, by use of homo- or hetero-bifunctional cross-linking reagents, a technique which was successfully used in the past for the identification of neigboring polypeptides of the photosynthetic bacterial reaction center and antennae [12,13].

The results presented here demonstrate that within intact thylakoids, the 32 kDa polypeptide assumes a configuration which prevents its cross-linking with any other thylakoid polypeptide, unless the structural integrity of the membrane is altered by treatment with detergents.

Materials and Methods

Prepatation of $^{35}SO_4^{2-}$-labeled thylakoids

Chlamydomonas reinhardtii y-1 cells were grown as previously described, using a mineral medium supplemented with sodium acetate as a carbon source [14]. In vivo pulse labeling was performed as described before, using resting cells and $^{35}SO_4^{2-}$ at a final concentration of 3–5 μCi/ml, and specific activities in the order of 300–800 μCi/μmol [15]. Incubation with radioactive sulfate was for 60 min in a temperature-controlled water bath (25°C). Illumination with white light (300–500 W/m^2) was provided as described below; then cold SO_4^{2-} was added to reduce the specific activity of $^{35}SO_4^{2-}$ to approx. 0.5 μCi/μmol, and incubation was further continued for 3.5 h [2,15] (chase). Thylakoids were prepared as described [15,16] and used either immediately or stored at −80°C.

Cross-linking and separation of thylakoid polypeptides

The thylakoids were washed twice by centrifugation at 5°C in 100 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 5 mM $MgCl_2$. Cross-linking was carried out by the addition of *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) dissolved in ethanol, or 3,3′-dithiobis(succinimidyl)propionate (DTSP) dissolved in dimethylsulfoxide (DMSO), or the water soluble 3,3-dithiobis(sulfosuccinimidyl)propionate (DTSSP) (Pierce, final concentrations, 0.5–1 mM). Incubation was carried out at 25°C for 15 min in the dark. In some experiments, glutaraldehyde (Merck) was used at 4°C for up to 20 min in the dark. In all cases the chlorophyll concentration was 200 μg/ml.

Resolution of various polypeptides and detection of the 32 kDa polypeptide and fragments derived thereof by trypsin treatment was performed by lithium-dodecyl-sulfate polyacrylamide gel electrophoresis (10–17.5%) in the presence of 4 M urea and autoradiography [10].

Photoinhibition and measurements of photosynthetic activity

Photoinhibition in vitro was done as described earlier [5], using chlorophyll concentrations of 200 μg/ml and exposing the thylakoid suspension to 1200 W/m^2 at 25°C for 30 min. Incubation was carried out in a water bath maintained at 25°C. White light was provided by a tungsten halogen

lamp and measured in the water bath at the level of the sample, with an YSI Kettering model 65 radiometer probe. For detergent treatment, thylakoids were incubated prior to or after cross-linking in the above buffer, with additions of octyl-β-D-glucoside (0–2.5% final concentration) during various times, at 25°C in the dark. Rates of electron flow using H_2O or diphenylcarbazide (DPC) as donors and dichlorophenol-indophenol (DCIP) or sodium-12-molybdosilicate (SiMo) as acceptors, were carried out as described earlier [4]. ^{3}H-Diuron binding was performed as described by Shochat et al. [17].

Immunoblotting

For detection of the 32 kDa polypeptide after transfer by electrophoresis to nitrocellulose paper, antibodies prepared against a 23 kDa fragment of the 32 kDa protein, kindly donated by Dr. J. Hirschberg, were used as described before [6] and detected by decoration with anti-rabbit IgG-alkaline phosphatase conjugate (Sigma A8025).

Trypsin digestion

Isolated thylakoids were suspended in phosphate buffer as above, at a final concentration of 200 μg chlorophyll/ml, and trypsin (Sigma, type XIII) was added (50 μg/ml). Incubation was carried out at 25°C for 20 min, and reaction was stopped, after centrifugation of the membranes in an Eppendorf microfuge, by the addition of the electrophoresis sample buffer.

Results

(a) Effect of the cross-linkers on electron flow and Diuron binding

In order to test the effects of cross-linking with SPDP and DTSP on the proteins of Photosystem II, electron flow from H_2O or DPC to SiMo (independent of Q_B) or DCIP (dependent on Q_B) and ^{3}H-Diuron binding were measured in cross-linked membranes. The results (Table I) show that cross-linking with SPDP does not inhibit electron flow from DPC via PS II reaction center to SiMo, while water-splitting and electron flow to DCIP via Q_B was significantly inhibited. At the same time, the number of Diuron binding sites was reduced about 3-fold, while no significant changes occurred in the Diuron binding constant (Table I).

When both reactive groups of SPDP were blocked prior to cross-linking by the addition of ethanolamine and *N*-ethylmaleimide, no effect was observed on the activities, as compared to appropriate controls. Blocking of the reagent reactive group, forming a covalent bond with NH_2-residues by addition of ethanolamine, had only a partial effect, whereas blocking the group reacting

TABLE I

EFFECT OF CROSS-LINKING REAGENTS ON PS II ACTIVITY

The incubation was carried out at 25°C for 15 min in a system containing 100 mM phosphate buffer (pH 7.5) 0.2 mg per ml Chl, 1 mM SPDP, 0.5 mM DTSP, 2 mM NEM or ETA. When added together, the cross-linkers were preincubated with NEM or ETA for 15 min. NEM, *N*-ethylmaleimide; ETA, ethanolamine. The 100% activities were 180 and 46 μmol substrate reduced per mg chlorophyll per h for DCIP and SiMo reduction, respectively.

Experiment (order of additions)	Percentage activity (μmol per mg Chl per h)			Chl/DCMU (M/M)	K_d (M)
	$H_2O \rightarrow$ DCIP	DPC → DCIP	DPC → SiMo		
Control	100	100	100	233	$9.5 \cdot 10^{-9}$
Control + NEM	68	52			
Control + ETA	61	70			
Control + SPDP	18	23	110	666	$6.3 \cdot 10^{-9}$
Control + ETA + SPDP	55	49			
Control + NEM + SPDP	0	15			
Control + ETA + NEM + SPDP	65	59	130		
Control + DTSP	15	16	45	130	$2.6 \cdot 10^{-8}$
Control + ETA + DTSP	63	48			

with SH-residues of the proteins by addition of *N*-ethylmaleimide caused a drastic reduction of electron flow from H_2O or DPC to DCIP.

When thylakoids were reacted with DTSP, a marked reduction was observed in the above activities and a somewhat lesser effect on the electron flow via PS II reaction center to SiMo. At the same time a slight reduction in the Diuron binding constant was observed, while the amount of Diuron bound relative to chlorophyll increased about 2-fold, probably by increase of the non-specific binding.

(b) Changes in the electrophoretic pattern of thylakoids polypeptides following treatment with various cross-linkers

Treatment of isolated thylakoids with hydrophobic (SPDP, DTSP) or hydrophilic cross-linkers (DTSSP, glutaraldehyde) results in an alteration of the thylakoid polypeptide pattern, as revealed by LDS-polyacrylamide gel electrophoreses, indicating extensive cross-linking. It appears that most of the cross-linked polypeptides form large complexes which are excluded by the stacking gel. Since reducing agents were not used in the solubilization step to avoid the cleavage of the disulfide bridge of the bifunctional reagents, the electrophoretic separation is rendered more difficult and the resolution of the individual polypeptides is poor as compared with that of samples treated with dithiothreitol or β-mercaptoethanol. Almost all the thylakoid polypeptides were affected by the cross-linkers used, as shown by the alteration of the Coomassie brilliant blue staining patterns. Among the polypeptides of the light-harvesting chlorophyll *a/b* complex (29, 26 and 24 kDa respectively), there seems to be a differential effect by the various cross-linkers. Among the cross-linkers used, SPDP generates a slightly different pattern of cross-linked polypeptides as compared with DTSP or DTSSP. Almost complete cross-linking was obtained by glutaraladehyde even at low concentrations (Fig. 1). However, an examination of the autoradiograms of LDS-polyacrylamide gel electrophoresis resolved polypeptides, shows that the 32 kDa polypeptide, identified

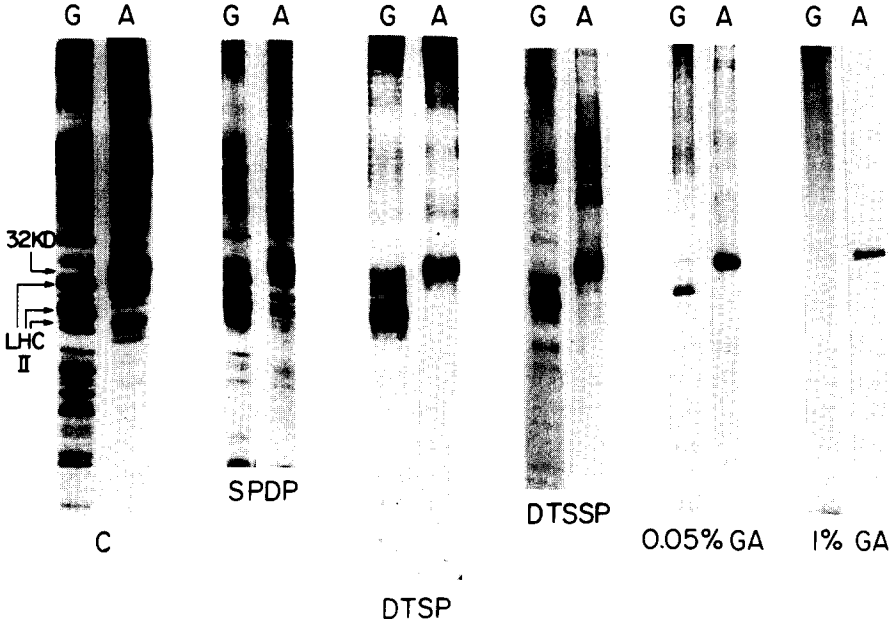

Fig. 1. Cross-linking of *Chlamydomonas* thylakoid polypeptides by hydrophobic (SPDP, DTSP) and hydrophilic (DTSSP, glutaraldehyde) cross-linkers. ^{35}S-labeled thylakoids were treated with various cross-linkers in the dark at 25°C for 15 min, and then the polypeptides were resolved by LDS-polyacrylamide gel electrophoresis. For additional details see Materials and Methods. In all cases, the 32 kDa polypeptide was not cross-linked with other thylakoid polypeptides. C, control, untreated thylakoids; G, stained gel; A, autoradiogram; LHC II, the chlorophyll *a/b*-binding polypeptides; the figure shows gel patterns obtained from various experiments.

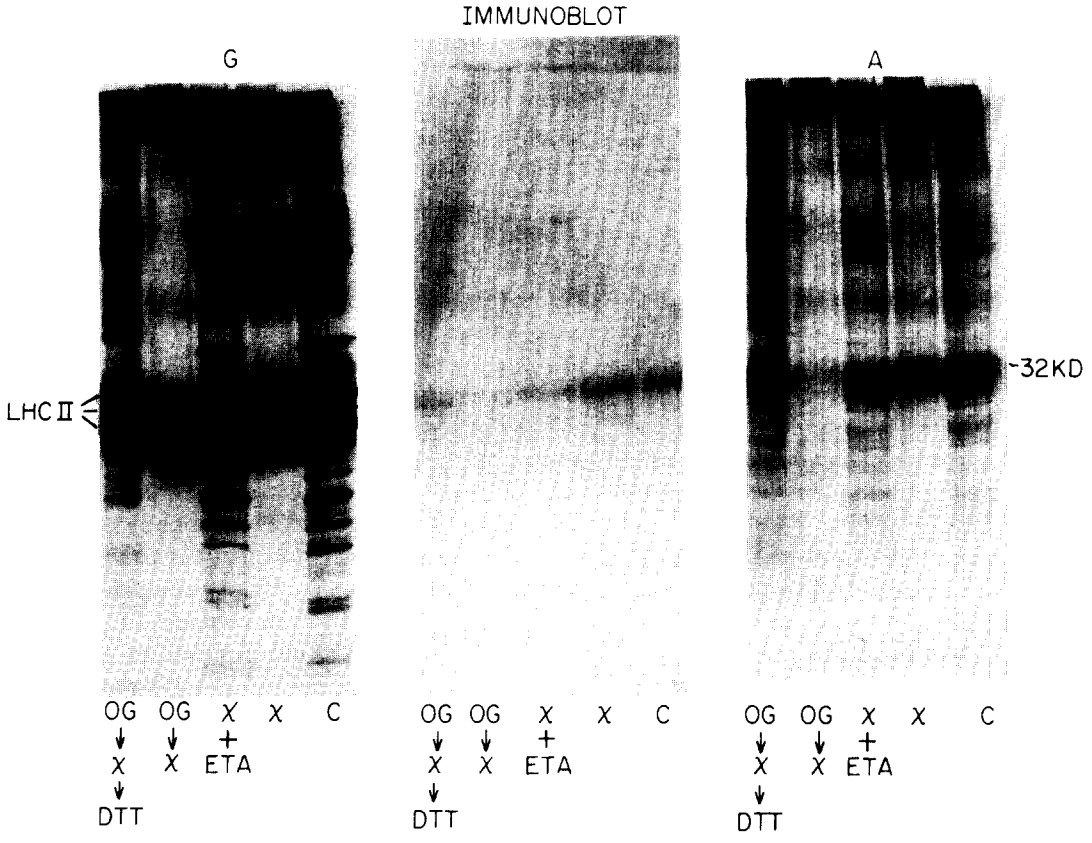

Fig. 2. Effect of β-D-octylglucoside on the cross-linking of the 32 kDa polypeptide by DTSP. ^{35}S-labeled thylakoids were reacted with DTSP (0.5 mM) for 15 min at 25°C, in the absence of χ) or after preincubation with 2 mM ethanolamine (χ + ETA) or after treatment with 2.5% β-D-octylglucoside (OG) for 60 min (OG → χ). After treatment with detergent, part of the thylakoids were subsequently incubated with 0.5 M dithiothreitol prior to electrophoresis (OG → χ → dithiothreitol); G, stained gel; A, autoradiogram; C, control, untreated membranes. For immunoblotting (central panel) the resolved polypeptides were transferred to nitrocellulose papaer, and the 32 kDa polypeptide was detected by specific anti-Q_B antibodies. Note the reduction of the 32 kDa polypeptide in the thylakoids cross-linked after treatment with detergent and its reappearance after cleavage of the dithiol link of the reagent by dithiothreitol in both autoradiogram and immunoblot.

from its high ^{35}S-labeling (Fig. 1) or reaction with antibody (see below), is practically unaffected by all the cross-linkers tested. Even treatment with 1% glutaraldehyde resulted only in a partial loss of this polypeptide (Fig. 1).

As opposed to these results, the 32 kDa polypeptide appears to be cross-linked and does not migrate to its usual position in the electrophoretic separation when cross-linking is performed after preincubation with β-D-octylglucoside (Fig. 2). Blocking of the reactive groups of DTSP with ethanolamine prevents cross-linking of the stainable polypeptides as well as that of the ^{35}S-labeled 32 kDa polypeptide. Pretreatment of the thylakoids with β-D-octylglucoside resulted in cross-linking of the 32 kDa-Q_B-protein also when all the other cross-linkers were used (data not shown). The high molecular-weight complexes formed by cross-linking in the presence of detergent, containing the 32 kDa polypeptide's radioactivity, can be at least partially cleaved following addition of dithiothreitol after treatment with DTSP, and the 32 kDa polypeptide is released and can be detected in its usual position on the electrophoretogram by autoradiography or reaction with specific antibodies (Fig. 2). The effect of various concentrations of detergent (0.25–1.5%) and time of incubation at higher detergent concentrations

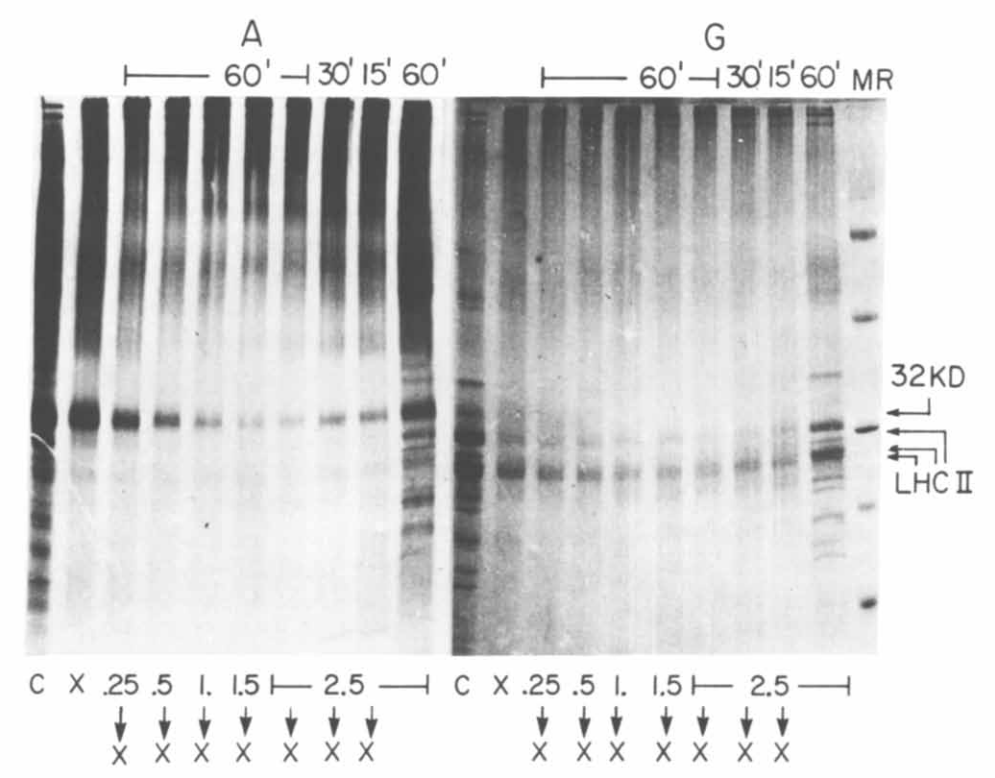

Fig. 3. Effect of β-D-octylglucoside concentration and time of incubation on the cross-linking of the 32 kDa polypeptide by DTSP. Same experimental conditions as in Figs. 1 and 2, C, control untreated thylakoids; X, thylakoids cross-linked with DTSP; numbers above, time of incubation in min, numbers below, detergent concentration (%); G, stained gel; A, autoradiogram.

(2.5%) are shown in Fig. 3. It is evident that a significant effect is already obtained at a detergent concentration of 0.25%; even at 10-fold concentrations, the effect is proportional to the time of incubation with β-D-octylglucoside (Fig. 3).

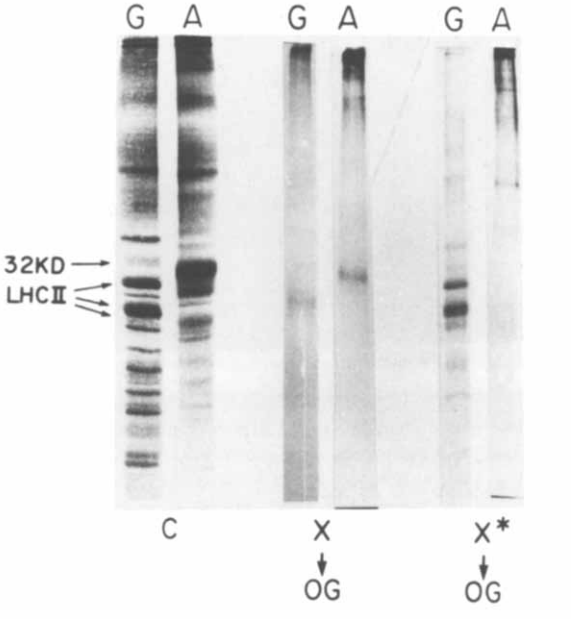

Fig. 4. Effect of β-D-octylglucoside on the 32 kDa polypeptide after treatment of thylakoids by DTSP or SPDP. ^{35}S-labeled thylakoids were incubated in the dark at 25°C for 15 min with DTSP (0.5 mM) or SPDP (1.0 mM), then washed extensively to remove all traces of unreacted reagent. The thylakoids were then incubated at 25°C with 2.5% detergent at a final concentration of chlorophyll of 600 μg/ml, and samples were resolved by LDS-polyacrylamide gel electrophoresis G, stained gel; A, autoradiogram; C, control, untreated thylakoids; X, X*, DTSP- and SPDP-treated samples, respectively; arrow, order of addition. Note the extensive reduction or complete loss of the radioactive band at 32 kDa in the detergent-treated samples (compared with Fig. 1). The figure is compounded from different experiments in which the degree of labeling of the 32 kDa polypeptide relative to the other membrane polypeptides was different.

(c) Effect of detergent treatment after cross-linking of thylakoid polypeptides with DTSP or SPDP

To test the possibility that the cross-linkers used in this work react with the 32 kDa polypeptide eventually forming monovalent links with some of the amino acid residues, thylakoids were first reacted with SPDP or DTSP, then washed extensively free of non-reacted cross-linkers, followed by treatment with β-D-octylglucoside. The rationale of this experiment is that, if monovalent links are generated within the 32 kDa polypeptide resulting in 'activated' molecules, they might further react with amino acid residues of other thylakoid polypeptides, following denaturation of the membrane by detergent treatment. The results of such an experiment show that monovalently cross-linked residues might be generated by SPDP but only to a limited extent by DTSP, as demonstrated by the complete loss of the ^{35}S-labeled 32 kDa polypeptide in the former case but only partial loss in the latter (Fig. 4, compare with Fig. 1).

(d) Effect of high light intensity (photoinhibition) and treatment with trypsin on cross-linked thylakoids

The data presented above show that the 32 kDa polypeptide is not cross-linked with other thylakoid polypeptides. However, it is possible that the bifunctional reagents interacted with the 32 kDa polypeptide, forming intramolecular bridges or, at least, reacted with single amino acid residues (arginine, cysteine) resulting in the formation of monovalent, active residues. It is known that treatment of thylakoids of both higher plants and *Chlamydomonas* with trypsin results in the cleavage of the 32 kDa polypeptide at specific sites and the formation of a 15.7–20 kDa trypsin fragment, depending on whether electrophoretic separation is performed in the presence or absence of urea [10,18,19]. Thus, one would expect that blocking of arginine residues by monovalent or bivalent cross-linking might result in an alteration of the trypsin activity on the 32 kDa polypeptide. To test this possibility, thylakoids were first treated

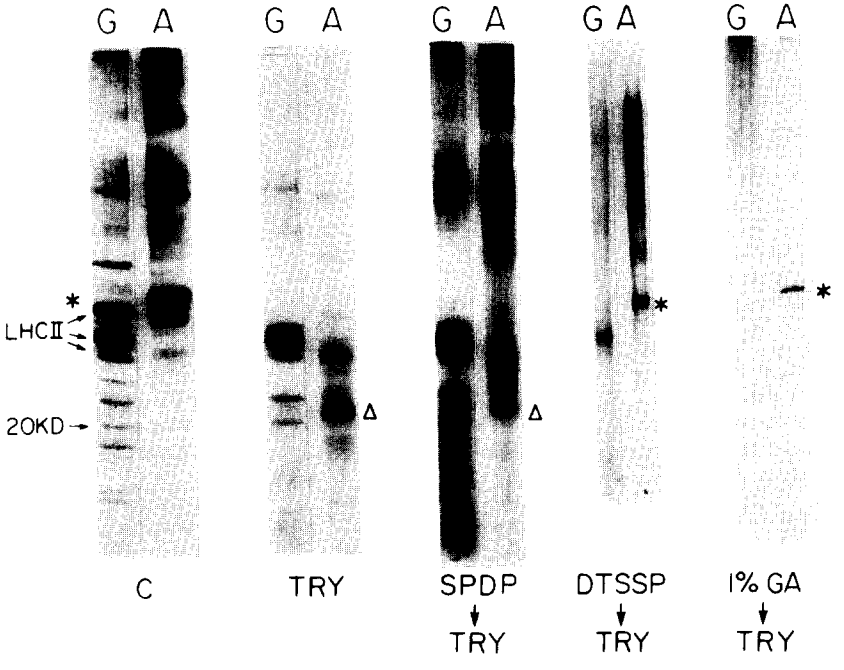

Fig. 5. Effect of cross-linking of thylakoid polypeptides on proteolysis by trypsin. ^{35}S-labeled thylakoids were treated with various cross-linkers, as indicated, at a concentration of 1 mM SPDP and DTSSP for 15 min at 25°C in the dark, or 1% glutaraldehyde for 20 min at 4°C in the dark. The cross-linked thylakoids were then washed free of the reagent and incubated with trypsin, as described. Samples of the entire incubation mixture were loaded and resolved by LDS-polyacrylamide gel electrophoresis. Note the disappearance of the radioactive band of 32 kDa (asterisk) and appearance of a heavily radioactive band at about 20 kDa in the trypsin-treated and SPDP cross-linked thylakoids only (Δ); G, stained gel; A, autoradiogram; TRY, C, trypsin-treated and control thylakoids, respectively.

with SPDP, DTSSP or glutaraldehyde before exposing them to the trypsin action. The results of such an experiment show that only in the SPDP-treated ^{35}S-labeled thylakoids, trypsin digestion generated a radioactively labeled peptide with an electrophoretic mobility corresponding to that of the trypsin fragment of 20 kDa obtained from control thylakoids, and a concomitant loss of the ^{35}S-labeled 32 kDa polypeptide was observed (Fig. 5). A similar trypsin fragment was not detected in thylakoids treated with DTSSP or glutaraldehyde (Fig. 5). Similar results were obtained with DTSP (data not shown). The fact that treatment with DTSP, a hydrophobic reagent, prevented the action of trypsin on the hydrophilic surface-exposed segment of the Q_B-protein, could be due to changes in the conformation of the protein hydrophobic segments embedded within the membrane lipid bilayer, which affected the orientation of the surface-exposed arginines (see also Fig. 4). Alternatively, cross-linking of the other thylakoid polypeptide by DTSP could result in the formation of large multi-protein complexes in close proximity to the Q_B-protein, which prevented the trypsin access.

As glutaraldehyde appears to cause almost complete cross-linking of all the stainable thylakoid polypeptides, and also reacts with the 32 kDa polypeptide, it could be expected that the glutaraldehyde-treated thylakoids lose their susceptibility to the light-induced degradation of the 32 kDa polypeptide. The results of such an experiment in which the effect of various glutaraldehyde concentrations or times of incubation were tested,

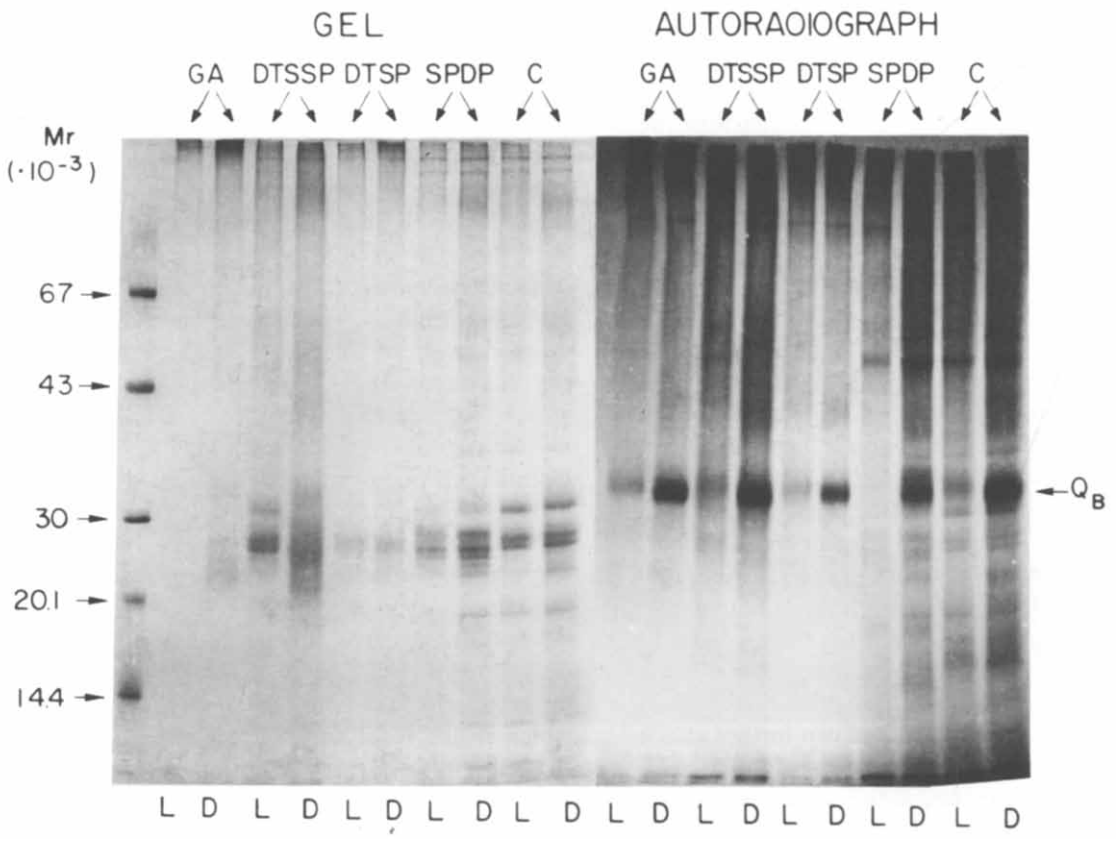

Fig. 6. Effect of cross-linking on the light-dependent degradation of the 32 kDa polypeptide. ^{35}S-labeled thylakoids were treated with cross-linkers (1 mM) at 25 °C in the dark for 15 min. The thylakoids were washed free of the reagents and exposed to 1200 W·m^{-2} white light at a chlorophyll concentration of 200 μg chlorophyll/ml at 25 °C for 20 min. Samples of the total incubation system were resolved by LDS-polyacrylamide gel electrophoresis. C, control, untreated thylakoids; PI, thylakoids exposed to high light intensity. Note the disappearance of the ^{35}S-labeled band of 32 kDa in all samples.

demonstrated that the light-induced degradation of the 32 kDa polypeptide does not seem to be inhibited in the glutaraldehyde-treated thylakoids at all concentrations (data not shown). Similar results were obtained with all of the reagents used in this study, which possess a lesser cross-linking activity on the thylakoid polypeptides (Fig. 6).

Discussion

The results presented in this work demonstrate that the 32 kDa herbicide-binding and fast turning-over polypeptide is not cross-linked to neighboring proteins, either by hydrophobic or hydrophilic cross-linkers of about 0.8–1.1 nm length nor by glutaraldehyde which may form bridges of various lengths [20]. The identification of the 32 kDa polypeptide is based on its fast labeling by $^{35}SO_4^{2-}$ [1,2,5,15] specific degradation in light exposed thylakoids in vitro [6] and reaction with specific antibodies. However, reduction of electron-flow activity and herbicide binding indicate that the cross-linkers might have reacted with the 32 kDa polypeptide possibly by formation of intramolecular bridges, or at least monovalent links with some of the arginine or cysteine residues of the polypeptides (see below).

The inability of all the reagents used to cross-link the 32 kDa polypeptide to other thylakoid polypeptides is not due to the absence of reactive groups such as lysine in the 32 kDa polypeptide of *Chlamydomonas* [21], since following denaturation of the membrane by high detergent concentrations, cross-linking is obtained. Furthermore, addition of the detergent after cross-linking and removal of the non-reacted reagent caused a partial or complete cross-linking of the 32 kDa polypeptide when DTSP or SPDP were used respectively, thus implying that at least some of the 13 arginine and/or 4 cysteine residues of this polypeptide [21] react with one of the functional group of the bivalent cross-linkers and can further attach to amino acid residues of other thylakoid polypeptides, following denaturation of the membranes by addition of the detergent. Since treatment of thylakoids with SPDP or DTSP causes extensive cross-linking of almost all membrane polypeptides, without cross-linking of the 32 kDa, we conclude that the detergent might primarily affect the lipid surroundings of the 32 kDa polypeptide and, directly or indirectly, the conformation of the polypeptide itself. This might result in a different orientation of the reactive amino acid residues which may come into proximity with similar groups of neighboring proteins, or, alternatively, affect the degree of nucleophylicity of $-NH_2$ or $-SH$ groups and enhance their reaction with the cross-linkers. Based on hydrophatic plots, Cramer et al. [22] concluded that this polypeptide crosses the membrane seven times, while exposing its carboxyl terminal and possibly also the amino terminal on the outer surface of the thylakoid. A different hydrophatic plot, representing the protein as crossing the membrane only four times, was proposed by Trebst [23]. According to Cramer et al. [22] and Edelman et al. [24], the surface-exposed segments contain arginine residues susceptible to proteolytic cleavage by trypsin and possibly to the cross-linkers as well, while at least two of the cysteine residues appear to be located within the hydrophobic phase of the membrane. The polypeptide is highly hydrophobic and therefore it could be expected that its conformation and relative position of the membrane-contained loops might be strongly influenced by the surrounding lipid phase.

Our results are consistent with the view that this conformation might be stabilized by some specific lipid-protein interaction, as reported also for other hydrophobic proteins such as rhodopsin which is also refractile to cross-linking by glutaraldehyde up to about 2% [25,26]. Alterations of this lipid phase might thus cause displacement of or rotation of the 32 kDa intramembrane segments and allow residues of arginine or cysteine to come into close proximity with similar amino acid residues of other polypeptides. However, it should be considered that a similar situation might also occur for other neighboring thylakoid polypeptides. The fact that DTSSP or glutaraldehyde, which might react with surface exposed 32 kDa segments, do not result in intermolecular bridges, further supports the view that indeed these segments are folded in such a way as to be unavailable within approx. 1 nm to arginine, lysine or cysteine residues.

The results obtained by trypsinization of cross-linked membranes indicate that SPDP might form

primarily monovalent links with amino acid residues of the 32 kDa polypeptide and be remote from the site of trypsin action (residues 225, 238 and 323) which do not prevent proteolysis and generation of the trypsin fragment, as reported before [18,19]. On the other hand, DTSP, DTSSP and glutaraldehyde might react with these surface exposed residues and prevent the action of trypsin. One of the striking results obtained int his work is that after reaction with SPDP and DTPSP or DTSSP, which cause an extensive cross-linking of most of the thylakoid polypeptides and affect the 32 kDa polypeptide as well, the latter is still susceptible to the process of light-dependent degradation, while electron flow, requiring the activity of the Q_B site, and the number of herbicide binding sites are specifically reduced. This would imply that the degradation induced by light does not involve the activity of a specific protease whose activity is triggered by an electron flow-induced modification of the Q_B protein, as suggested earlier [6]. Such a degradation could be due to the formation of free radicals generated by excitation of the pigment bed of the membrane. The fact that even cross-linking with 1% glutaraldehyde which inhibits almost completely photosynthetic electron flow (data not shown), did not abolish the light-induced degradation, is in agreement with this explanation. As opposed to that, DCMU which completely blocks electron flow via Q_B, offers partial protection against photoinhibition both in vivo [4] and in vitro (Ref. 6). Further experimental data are needed before these phenomena can be understood.

Based on previously published results [7] and those presented here, we would like to suggest, as a working hypothesis, that a specific lipid annulus participates in the formation of a functional 32 kDa-Q_B site by inducing its intramembrane conformation in such a way as to prevent its close proximity with other polypeptides of the PS II reaction center and confines semiquinone radicals or other free radical species generated by light excitation within the 32 kDa-lipid complex, which might explain its susceptibility to the light-dependent degradation. The presence of such a lipid annulus should not prevent the access of the oxidized plastoquinone from the lipid phase of the membranes or Q_B interaction with Q_A sites, but might hinder the diffusion of charged free radicals formed within the quinone binding site or the hydrophobic phase of the 32 kDa-lipid complex. If this were the case, one could consider the 32 kDa polypeptide as a 'suicide' complex devised to act both as an electron carrier and free radical scavenger of the PS II complex. Based on the amino-acid homology of the Q_B and D_2 proteins to the L and M subunits of bacterial reaction centers and their spatial orientation as emerging from X-ray diffraction analysis of *Rps. viridis* reaction center crystals [27], it has been recently proposed that the Q_B (or D_1) or D_2 proteins might be organized in a similar way and form the reaction center II of oxygenic chloroplasts [28].

Although our results do not necessarily argue against this hypothesis, they indicate that due to interactions with a specific hydrophobic environment, the Q_B protein's –SH or $-NH_2$ groups are not sufficiently close to other proteins to be cross-linked by the reagents used in this work. One should also consider that the L and M subunits of the bacterial reaction centers of *Rps. capsulata* and *Rps. sphaeroides* [29,12] are cross-linked to each other when the intact chromatophores are treated with DTSP. Further probing with additional cross-linkers of increasing lengths might shed light on the immediate surroundings of the 32 kDa-Q_B-protein, including other proteins and its lipid environment, and the mechanism of its function and light-dependent degradation.

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