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The Rapidly-Metabolized Herbicide Binding Protein of the Thylakoids: Relationship to Photosynthesis and Crop Protection

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Title

THE RAPIDLY-METABOLIZED HERBICIDE BINDING PROTEIN OF THE THYLAKOIDS:

Relationship To Photosynthesis and Crop Protection

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ABSTRACT

The photosystem II (PS II) reaction center is a quinone-reductase. One of its components, the D1 protein, rapidly turns over in the light but is stable in the dark. Degradation is driven by visible, far red and UV radiation. Two major steps have been distinguished: 1. a photoreceptor, characterized by its specific action spectrum, is activated by radiation; 2. the energized photoreceptor, directly or indirectly, activates a cleavage site resulting in the appearance of a specific breakdown product. We have identified elements of the D1 protein which comprise part of the degradation signal pathway. These elements form part of the quinone binding niche of the protein. The quinone acts as the photoreceptor for UV-B-driven D1 degradation. Mixing UV-B and visible light (to approximate sunlight) stimulates breakdown of the D1-D2 protein heterodimer of the reaction center in a synergistic manner. This identifies PS II as a major specific target of UV-B radiation.

As regards the dynamics of D1-PS II cycling, a PS II reaction center particle from the stromal lamellae was isolated. It contained the same proteins found in the granal PS II reaction centers. However, the cytochrome b_{559} content was half that of the granal centers. Pigment composition, fluorescence emission and excitation spectra all indicate a fully functional PS II particle in the stromal lamellae.

ORIGINAL OBJECTIVES

1. UV-activated turnover of D1:

To identify the photoreceptor(s) for D1 degradation in the visible and UV regions of the spectrum; to determine the role of free radicals in the degradation process; to quantitate the phenomenon of UV-activated D1 turnover.

2. Dynamics of D1-PSII cycling:

To determine the temporal and spacial characteristics of PSII reaction center disassembly and reassembly; to reconstitute processing and translocation of D1 *in vitro*.

RESEARCH FINDINGS

Chapter 1. Introduction

Overview

Photosystem II (PS II) of oxygenic phototrophs is a highly structured protein-pigment complex consisting of a water splitting system, a light-harvesting chlorophyll protein complex and a reaction centre (1, 2). The core of the PS II reaction centre consists of the D1, D2 and cytochrome b559 proteins (3, 4). Based on the resolved crystal structure of the functionally-analogous reaction centre of photosynthetic bacteria (5), and molecular characterization of herbicide resistant mutants (2), a structure for the PS II reaction centre has been proposed (6, 7).

The D1 protein is unstable in the light, turnover occurring at low light intensities with rates exceeding those for other proteins (14-16). A cleavage site for light-dependent degradation of the protein has been proposed in the loop between helices 4 and 5, somewhere between Arg238 and Ile248 (17). This site is in close proximity to the QB binding niche (6,7). It has been hypothesised that QB-anion radicals, normally formed during photosynthetic electron flow, are damaging species (8). To test this hypothesis, we tackled the question whether photosynthetic electron transport and D1 degradation are coupled in visible light, where the same photosensitizer (chlorophyll) is postulated to trigger them both (18). We used a variety of PS II inhibitors to block PS II driven electron flow. These inhibitors act by displacing QB from its site on the D1 protein (9-13). The most obvious result is an uncoupling between inhibition of photosynthetic electron transport and D1 degradation. For example, BNT inhibited D1 degradation with a considerable lower efficiency than it did electron flow. On the other hand, diuron inhibited both

processes with similar concentration dependence. We concluded that inhibition of D1 protein degradation by PS II inhibitors in visible light is not simply related to impairment of electron flow and displacement of plastoquinone. This work has been described in an article entitled "Differential sensitivity of D1 protein degradation and photosynthetic electron flow to photosystem II herbicides" (19). In an additional article entitled "PS II inhibitor binding, QB-mediated electron flow and rapid degradation are separable properties of the D1 reaction centre protein" (20) we further elaborated the uncoupling between PS II electron flow and D1 degradation by showing additional inhibitor-specific effects on D1 degradation, far red-driven D1 degradation, and white light-driven D1 degradation in mutants having a non-functional PS II.

The research described above, did not support the concept that quinone anion radicals act as damaging species which cause D1 degradation. Instead, the data suggested that specific (stretches of) residues of D1 are involved in the control of stability and that PS II inhibitors may interact with these regions. Not all PS II inhibitors do so, some compounds may not touch the regions of the QB-niche involved in the control of D1 stability. For example, bromonitrothymol (BNT) normally does not inhibit D1 degradation, however we found that some substituted nitrophenols were very efficient inhibitors of D1 degradation. Thus, the introduction of additional protein-inhibitor interactions resulted in inhibition of D1 degradation. We have related these additional protein-inhibitor interactions to a stretch of residues between helices D and DE, by comparing effects of PS II inhibitors on D1 degradation, and D1 trypsinization and by measuring binding in a series of site directed mutants. This work is described in the chapter entitled "Interactions of PS II inhibitors with the extended loop between helices D and DE of the D1 protein control the rapid degradation of D1 in vivo".

The differential effects of PS II inhibitors on D1 degradation were explored to yield information on the behaviour of inhibitors in situ. Co-analysis of electron flow and D1 degradation in the presence of diuron and/or BNT, visualized inhibitor displacement at the Qb-niche in intact plants. This work is described in the chapter entitled "Direct reporting on binding competition between inhibitors at the QB-niche of photosystem II in an intact higher plant".

A separate aspect of D1 instability is the degradation of this protein at UV-B wavelengths (280-320 nm). D1 protein degradation under these conditions has been extensively studied and turns out to be strikingly different from white-light driven degradation. we describe this work in the chapter entitled "The D1-D2 heterodimer of photosystem II is a major target of UV-B radiation". We focussed in this work on the following three themes: 1) the phenomenon of UV-B driven protein degradation (specificity, and flux density dependence), 2) the relevance of the UV-B driven degradation of the D1 and D2 proteins, considering potential increases in UV-B radiation reaching the biosphere, 3) the mechanism of UV-B driven degradation. The conclusion of this work is that the D1-D2 heterodimer of PS II is a specific target for UV-B radiation. We conclude that semiquinones act as photosensitizers in UV-B.

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Chapter 2. On the *in vivo* degradation signal pathway for the D1 protein

Introduction

Photosystem II (PS II) of oxygen evolving phototrophs is a highly structured protein-pigment complex consisting of a water splitting system, a light harvesting chlorophyll protein complex and a reaction centre (Murata and Miyao, 1985; Rochaix and Erickson, 1988). Based on analogy with the crystallized reaction center from purple bacteria, it has been proposed that the core of the PSII reaction center is a heterodimer consisting of the D2 and D1 proteins (Deisenhofer *et al.*, 1984; Nanba and Satoh, 1987; Marder *et al.*, 1987). These are the apoproteins of the electron transport chain intermediates QA and QB, respectively (Rochaix *et al.*, 1984). DNA cloning and sequencing has revealed a high degree of evolutionary conservation of the structure of the D1 and D2 proteins (Mattoo *et al.*, 1988). This is reflected in similar kinetics of QA to QB electron flow (Jansen and Pfister, 1990) and similar D1 protein metabolism throughout a wide range of oxygen evolving species (Hoffman-Falk *et al.*, 1982).

The D1 protein is unstable in the light and turns over more rapidly than other PS II proteins (Mattoo *et al.*, 1984; Gaba *et al.*, 1987; Gounaris *et al.*, 1987). Different photosensitizers are involved in this process in the visible and ultraviolet regions of the spectrum (Greenberg *et al.*, 1989). However, the mechanism(s) leading to degradation is not yet understood. The involvement of quinone (Kyle, 1985; Greenberg *et al.*, 1989), chlorophyll (Thompson and Brudvig, 1988) or hydroxyl radicals (Sopory *et al.*, 1990;

Richter *et al.*, 1990), as well as double-reduced QA (van Miegheem *et al.*, 1989) or QB-niche occupancy (Trebst *et al.*, 1988) has variously been hypothesized. Experiments *in vivo* reveal a 23.5 kDa breakdown product of the D1 protein sufficiently meta-stable to be identified by radiolabeling and immunological techniques (Greenberg *et al.*, 1987). Proteolytic mapping of this fragment suggests a cleavage site for light-dependent degradation of the protein in the region between helices D and E, particularly in the extended D-DE loop between R₂₃₈ and I₂₄₈ (Greenberg *et al.*, 1987). Experiments *in vitro* led to equivocal results: potential cleavage sites are suggested in the D-DE loop (Trebst and Depka, 1990; Shipton *et al.*, 1990) on the one hand, and between helices A and B on the other (Barbato *et al.*, 1991).

A wide range of inhibitors interrupts electron flow from PSII to the plastoquinone pool by displacement of liganded QB from its niche on the D1 protein (Velthuys, 1981; Arntzen *et al.*, 1982; Oettmeier and Soll, 1983; Vermaas *et al.*, 1983a; Vermaas *et al.*, 1983b). These PS II inhibitors have overlapping, but resolvable, binding sites on the D1 protein between helices D and E (Trebst and Draber, 1979; Trebst, 1986; Trebst, 1987). There is no simple relationship between herbicide binding, visualized as inhibition of electron transport, and D1 degradation. While DCMU and ioxynil inhibit linear electron transport and the rate of D1 degradation in parallel (Jansen *et al.*, 1992), bromoxynil inhibits the former more strongly and dinoseb, the latter (Jansen *et al.*, 1990). Thus, different PS II inhibitors having distinct binding interactions within the QB niche, affect D1-protein degradation differently.

The pattern of inhibition of a biological process by a series of inhibitor analogues may help identify biochemical (or structural) elements responsible for the inhibition. In this study, we analyse the effects of a variety of PS II inhibitors on D1 protein degradation. Our approach integrates several techniques: *in vivo* D1 degradation, *in vitro* D1 trypsinization and PS II inhibitor binding in wild type and inhibitor resistant strains. The results show that inhibitor-protein interactions which impede D1 degradation resemble those which impede D1 trypsinization and can be related to specific residues but not to others. These correlations support the involvement of the D-DE loop region in the rapid degradation of the protein.

Materials and methods

Turnover experiments were performed using axenic cultures of *Spirodela oligorrhiza* (Kurtz) Helgm, grown phototrophically under cool white fluorescent lamps. *In vivo* protein degradation assays were carried out as previously described (Marder *et al.*, 1986). Plants were radiolabeled with [^{35}S]methionine for 3 hours under $25 \mu\text{E.m}^{-2}.\text{s}^{-1}$ white light and chased either in the presence or absence of inhibitors under low light conditions ($6 \mu\text{E.m}^{-2}.\text{s}^{-1}$ cool white fluorescent light). To stop the chase reaction, the medium was drained and the plants were kept frozen until homogenization. Membrane proteins were separated by SDS-PAGE and visualized by autoradiography (Marder *et al.*, 1986). The rate of degradation of the D1 protein, relative to stable LHCP, was quantified by microdensitometry (Greenberg *et al.*, 1987).

Relative quantum yields of oxygen evolution were measured *in vivo* by photoacoustic spectroscopy (Bults *et al.*, 1982). The measuring light ($15 \mu\text{E.m}^{-2}.\text{s}^{-1}$) was modulated at a frequency of 15 Hz. Signals were processed by a lock-in amplifier (SR 530, Stanford Research systems, U.S.A.) using vectorial analysis (Poulet *et al.*, 1983). Under the low light conditions used, PS II inhibitors did not noticeably damage the photosynthetic apparatus as could be seen from the constant rates of oxygen evolution and D1 degradation during the chase experiment. Relative rates of photosynthetic electron flow and relative rates of D1 degradation

were plotted versus inhibitor concentration. The concentration reducing oxygen evolution with 50% (I_{50}) was determined, and the relative rate of D1 degradation at this concentration was assessed.

Trypsinization experiments were performed *in vitro* using freshly isolated spinach thylakoids, as previously described (Trebst *et al.*, 1988). Thylakoids, containing 300 μ g chlorophyll in 3 ml buffer (300 mM MES, pH 6.5 and 6 mM $MgCl_2$) were incubated at room temperature with 100 μ g trypsin (Boehringer) for 7 minutes. The reaction was stopped by adding aprotinin (1.5 mg/10 ml) (Trasylol^R Bayer). Membrane proteins were separated by SDS-PAGE and visualized by Western blotting (Trebst *et al.*, 1988). Alternatively (Fig. 2 only), spinach leaves were labeled with [^{14}C]leucine prior to thylakoid isolation, and separation of membrane proteins was visualized by autoradiography.

The (inhibition of the) photosynthetic activity of isolated spinach thylakoids was measured with a liquid phase oxygen electrode using ferricyanide as electron acceptor (Trebst *et al.*, 1988). Relative rates of photosynthetic electron flow and relative rates of D1 trypsinization were plotted versus inhibitor concentration. The lowest concentration which abolishes oxygen evolution was determined, and the relative rate of D1 trypsinization at this concentration was assessed.

Chlamydomonas wildtype and mutant strains were grown mixotrophically in the light. Cells were harvested in the exponential growth phase and thylakoids were isolated as reported (Wildner *et al.*, 1990). I_{50} -values for inhibition of uncoupled electron transfer from H_2O to DCPIP were determined. The V₂₁₉I and F₂₅₅Y mutants were originally isolated by Galloway and Mets (1984). The S₂₆₄A, A₂₅₁V and L₂₇₅F mutants were isolated by Wildner *et al.* (1990).

Substituted nitrophenols were kindly provided by Dr. W. Draber, Forschungszentrum Bayer AG, Monheim. Their inhibitory effects on PS II have been described (Trebst and Draber, 1979). An extensive report on the effects of substituted nitrophenols in different *Chlamydomonas* mutants is being prepared (W. Draber, U. Hilp and A. Trebst, in preparation).

Results

Some PS II inhibitors block D1 protein degradation, while others do not (Table 1). This is shown visually in the pulse-chase experiment of Figure 1. The triazine terbutryn impedes degradation of the D1 protein while the nitrophenol BNT has no such effect. Differences in functional behaviour have led to a classification of PS II inhibitors as either classical or phenolic (Trebst and Draber, 1986). We find that classical inhibitors (e.g., ureas, triazines) efficiently block D1 degradation (44% inhibition at the I_{50} for PS

II electron transport; Table 1) while phenolic ones (e.g., cyano-, nitro-, dinitrophenols) vary greatly in their effect (from 10% to 67% inhibition at the I_{50} ; Table 1). So, the observed inhibitor effects on D1 degradation cannot be accommodated by this simple grouping into two large inhibitor families.

Neither can a more specific classification based on chemical properties of inhibitors. We assayed three pairs of chemically closely related inhibitors for their effects on D1 protein degradation. The triazines atrazine and terbutryn affect D1 degradation similarly (Table 1). However, the cyanophenols bromoxynil and ioxynil which have different substituents at positions 6 and 2 of the phenyl ring, differ in their effects on D1 degradation. Ioxynil (R^6 and R^2 = iodo) is much more effective in inhibiting D1 degradation compared to bromoxynil (R^6 and R^2 = bromo) (Table 1). Also, the dinitrophenols dinoseb and DNOC which differ only in the size of their side chain at position 6 of the phenyl ring, have strikingly different effects on D1 degradation. Dinoseb (R^6 = 1-methyl-propyl) is an effective inhibitor while DNOC (R^6 = methyl) hardly affects the rate of D1 degradation (Table 1).

Based on the results in table 1, we speculated that the dimensions of the side chain at position 6 of the phenyl ring determine the effectiveness of cyanophenol and dinitrophenol PS II herbicides in inhibiting D1-degradation. This prediction was tested using a number of R^6 -substituted analogues of the nitrophenol BNT. The relative rates of D1 degradation and PS II oxygen evolution as a function of inhibitor concentration are given in

Fig. 2 for analogues with relatively small, moderate and big R⁶ side chains. A relative increase in inhibition of D1 degradation with increasing size of the R⁶ side chain is clearly evident. Table 2 lists the percent inhibition of D1 degradation for seven nitrophenol analogues at their I₅₀ for PS II oxygen evolution. BNT, with its isopropyl side chain at position 6, is a particularly poor inhibitor of D1 degradation. Replacement of the isopropyl group by increasingly more bulky groups (R⁶ = tert.-butyl, cyclic-pentyl, phenyl, cyclic-hexyl, or benzyl) results in increasingly more potent inhibitors of D1 degradation. We interpret these results to mean that by expanding specific side chains, one can introduce additional herbicide-protein interactions in the QB-niche, which can lead to inhibition of D1 degradation.

An attempt was made to localize the inhibitor-protein interaction(s) affecting D1 degradation. In isolated photosynthetic membranes, the D1 protein has major trypsinization sites in the D-DE loop region (Marder *et al.*, 1984). The PS II inhibitor DCMU, blocks *in vitro* trypsinization (Mattoo *et al.*, 1981; Trebst *et al.*, 1988) and *in vivo* degradation of the D1 protein (Mattoo *et al.*, 1984; Jansen *et al.*, 1992). Table 1 shows that a correlation between these two processes holds for seven of eight inhibitors investigated: those effective in blocking D1 degradation are usually effective in blocking D1 trypsinization, and *vice versa*.

The strength of this correlation was tested using the series of BNT analogues shown in table 2. Conditions for trypsinization of spinach thylakoids were chosen such that disappearance of the D1 protein band

following SDS-PAGE provides a semiquantitative measure of cleavage at R₂₃₈ with minimal cleavage at R₂₂₅ (Fig. 3). The relative rate of trypsin cleavage of the D1 protein at R₂₃₈, and PS II oxygen evolution as a function of inhibitor concentration are given in Fig. 4 for analogues with relatively small, moderate and big R⁶ side chains. A clear increase in inhibition of D1 trypsinization with increasing size of the R⁶ side chain is evident. Table 2 summarizes the results for the series of seven nitrophenol analogues. BNT does not markedly affect D1 trypsinization (Trebst *et al.*, 1988; Trebst, 1991). Replacement of the R⁶ iso-propyl group by a tert.-butyl group still does not result in effective inhibition of D1 trypsinization. However, replacement by bulkier groups like cyclic-pentyl, phenyl, cyclic-hexyl or benzyl results in increasingly potent inhibitors of D1 trypsinization (Table 2). Changes made at other positions in the BNT molecule (e.g. the presence of a methyl group at R³ [Table 2], or the identity of the halogen substituent at R² [data not shown]), do not affect trypsinization of D1. Thus, these data support an analogy between *in vivo* D1 degradation and *in vitro* D1 trypsinization.

Several herbicide resistant mutants in *Chlamydomonas* have amino acid changes in the D1 protein between helices D and E (Wildner *et al.*, 1990). We analysed five mutants for their tolerance to the BNT-analogue series. Mutant L₂₇₅F varied over a 20-fold range in tolerance response to various analogues but without any recognizable pattern. The pattern in the response of mutant A₂₅₁V results from the presence of a methyl group at position R³ in BNT and a tert.-butyl analogue (similar elevated values were obtained in three other inhibitor pairs of R³-methyl versus R³-hydrogen

for this mutant [data not shown]) and is not part of a response pattern to changes at position R⁶ in this mutant. Neither are correlative response patterns discernable for mutants S₂₆₄A or F₂₅₅Y (Table 2). However, for mutant V₂₁₉I there is a clear pattern of increasing inhibitor tolerance with increasing size of the R⁶ substituent, which correlates well with the the patterns of inhibition of D1 degradation and trypsinization (Table 2). Additionally, the observed pattern of inhibitor tolerance allows for the first time to orient phenol type inhibitors in the Q_B site.

DISCUSSION

We have provided direct evidence that PSII inhibitors control D1 degradation via interactions with specific parts of the Q_B niche. This was arrived at by parallel measurements of D1 degradation, D1 trypsinization and inhibitor tolerance for a series of inhibitor analogues and mutants. Inhibition of D1 degradation *in vivo* and D1 trypsinization *in vitro* correlated well. In addition, we note the parallel effect of light versus dark on these processes (Mattoo *et al.*, 1981). Furthermore, D2 trypsinization and D2 degradation are affected in parallel (i.e., both are inhibited by atrazine and diuron, but not by BNT [Trebst *et al.*, 1988; Jansen *et al.*, in preparation]). Thus, while the inhibitor effects on the two processes are not identical (e.g., effects of dinoseb [Table 1]), understanding the inhibition of one is relevant to an understanding of the other.

The biological importance of the observed analogy is that in the absence of PSII inhibitors, quinones might similarly control D1 stability. It has been shown that the presence of oxidized plastoquinone retards trypsinization of the D1 protein in the dark (Trebst *et al.*, 1988). By analogy, we predict a similar inhibitory effect of oxidized plastoquinone on D1 degradation *in vivo*. The plastoquinone pool is supposedly oxidized under far red radiation. Yet, the D1 protein is rapidly degraded when exposed to far red light (Gaba *et al.*, 1987; Jansen *et al.*, 1992). To accommodate this observation, we suggest that D1 degradation occurs when the Q_B niche is empty. Due to the dynamic equilibrium between oxidized Q_B and plastoquinone, the Q_B niche may transiently be empty. Yet, also light is a requirement for degradation, as the D1 protein is stable in the dark. Therefore, we suggest a two step mechanism, an empty niche is potentially primed for degradation, but degradation occurs only if the relevant photosensitizer is activated. We arrived at the same conclusion independently, via our results with BNT. If BNT is present in the Q_B niche, the D1 protein can potentially be degraded (Fig. 1.), but this occurs only in the presence of light. Gong and Ohad (1991) also concluded that the ligand bound to the Q_B site controls D1 stability. Yet, based on studies of D1 degradation in *Chlamydomonas* mutants impaired in plastoquinol oxidation, these authors suggest that the presence of Q_B in the niche is essential for D1 degradation.

A second aspect of the analogy between D1 degradation and D1 trypsinization concerns the position(s) within the Q_B niche which are involved. Conditions for trypsinization were chosen such that

disappearance of the D1 protein band provides a semiquantitative measure of cleavage at R₂₃₈. The presence of the proposed D1 cleavage site in the region close to R₂₃₈ (Greenberg *et al.*, 1987), which was based on work done *in vivo*, can easily be accommodated by our data. The identification of R₂₃₈ is also in agreement with *in vitro* work, by Shipton *et al.* (1990) and Trebst and Depka (1990). Although, the cleavage site identified by Trebst and Depka (1990) may be more related to degradation in UV.

Binding of the "inhibitory" R⁶ side chain was related to residue V₂₁₉ but not to residues L₂₇₅, S₂₆₄, F₂₅₅ and A₂₅₁. The correlation of R⁶ side chain dimensions with tolerance in a V₂₁₉I mutant and with inhibition of D1 degradation and trypsinization specifically identifies the extended loop between helices D and DE as being involved in rapid light-dependent D1 degradation. The parallel identification of both V₂₁₉ and R₂₃₈ would suggest that these are neighbouring residues. This fits with the identification of Y₂₃₇, by azidomonuron tagging, as being part of the inhibitor binding domain (Dostatni *et al.*, 1988). Yet, it can not be excluded that the effect of PS II inhibitors on R₂₃₈ is indirect and relayed via either neighbouring residues in the loop between helices D and DE or even via stretches of residues further apart. Indeed inhibitor effects may extend as far as the D2 protein as shown by the inhibitory effects of diuron and atrazine on trypsinization of the D2 protein (Trebst *et al.*, 1988). Yet, irrespective of the signal transduction pathway, the data show that the effects of PS II inhibitors on the D-DE loop control D1 degradation. Recently a novel D1 cleavage site was proposed to occur in the hydrophilic loop connecting transmembrane helices A and B (Barbato *et al.*, 1991). In this

work isolated PS II reaction centres were exposed to photoinhibitory light. We note that the Q_B pocket of such isolated PS II reaction centres has lost its ability to bind PS II inhibitors with high affinity (Giardi *et al.*, 1988). It is unclear whether under these conditions the Q_B-niche still controls D1 degradation. The relation between the processes of photoinhibition of isolated PS II reaction centres and of D1 degradation *in vivo* under limiting light conditions remain to be shown.

We have related inhibition of both D1 degradation and D1 trypsinization to interactions of the R⁶ side chain of the cyanophenols, nitrophenols and dinitrophenols with residues in the loop between helices D and DE. All BNT analogues used, have similar pI₅₀-values for inhibition of photosynthetic electron flow (Table 2). Thus, side chains do not greatly contribute to binding and protein-side chain interactions are probably energetically weak. This might have been predicted independently from the chemical character of some of these side chains. Thus, the strong effects of specific BNT analogues on D1 degradation are due to weak hydrophobic or steric interactions. Therefore, we propose that PS II inhibitors, affect D1 degradation by affecting the accessibility of the protein towards either proteases (Virgin *et al.*, 1991) or radicals (Sopory *et al.*, 1990; Richter *et al.*, 1990).

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INHIBITOR	INHIBITION of	
	D1-degradation (%)	D1-trypsinization (+/-)
none	0	-
Classical		
<u>Triazines</u>		
atrazine	44	+
terbutryn	44	+
<u>Ureas</u>		
diuron	44	+
Phenolic		
<u>Cyanophenols</u>		
bromoxynil	10	-
ioxynil	60	+
<u>Dinitrophenols</u>		
DNOC	19	-
dinoseb	67	-
<u>Nitrophenols</u>		
BNT	10	-

Table 1

INHIBITOR				% INHIBITION		INHIBITOR TOLERANCE					
Backbone	R ⁶	R ³	pI ₅₀	D1 degr.	D1 tryp.	O ₂ -evolution (I ₅₀ mutant/I ₅₀ WT)					
					R ₂₃₈	V _{219I}	S _{264A}	A _{251V}	F _{255Y}	L _{275F}	
	OH	iso-propyl	7.1	10	12	2.0	0.4	8.0	0.6	1.6	
	Br	tert.-butyl	7.1	31	0	2.5	1.0	4.0	4.0	15.8	
	R ⁶	tert.-butyl	7.0	25	0	4.0	0.3	1.3	1.0	25.4	
		cycl.- pentyl	7.2	61	56	8.0	0.6	0.8	1.3	3.2	
		cycl.- hexyl	7.1	63	49	40.0	0.8	0.8	6.3	10.0	
		phenyl	7.2	82	62	20.0	0.5	0.2	2.5	1.6	
		benzyl	7.0	88	90	47.6	0.4	0.6	1.0	1.3

Table 2

Legends

Fig. 1. Light-dependent degradation of the D1 protein. *Spirodela* plants were pulse-labeled with [^{35}S]methionine, washed and transferred to chase medium containing methionine and PS II inhibitors as indicated. Chase times are given above each lane. Proteins were analysed by SDS-PAGE and autoradiography. Concentrations of terbutryn and BNT were respectively 3 μM and 1 μM . These concentrations reduced quantum yields of oxygen evolution to 15 to 20% of the non-inhibited control.

Fig. 2. Effects of three BNT analogues on the rate of D1 degradation and on the quantum yield of oxygen evolution. Compounds used, were respectively 6-isopropyl-3-methyl-bromonitrophenol (BNT), 6-pentyl-bromonitrophenol and 6-benzyl-bromonitrophenol. D1 degradation rates and quantum yields of oxygen evolution were determined using respectively pulse-chase experiments and photoacoustic spectroscopy as described in Materials and Methods. The data plotted, represent averages. All values are normalized to 100% in the absence of inhibitor. SE are shown ($n = 12$).

Fig. 3. Trypsinization of the D1 protein. Spinach leaves were pulse-labeled with [^{14}C]leucine, thylakoids were isolated and trypsinized as detailed in Materials and Methods. The time of incubation with trypsin is given beneath each gel lane. Proteins were analysed by SDS-PAGE. Gels were loaded on an equal chlorophyll content and the separated proteins were visualized by autoradiography.

Fig. 4. Effects of three BNT analogues on the rate of D1 trypsinization and on the rate of oxygen evolution. Compounds used, were respectively 6-isopropyl-3-methyl-bromonitrophenol (BNT), 6-pentyl-bromonitrophenol and 6-benzyl-bromonitrophenol. *In vitro* rates of D1 trypsinization and of oxygen evolution were determined by respectively Western blotting and a liquid phase oxygen electrode as described in Materials and Methods. The data plotted, represent averages. All values are normalized to 100% in the absence of inhibitor.

Fig. 1

Fig. 3

Chapter 3. Using D1 degradation as a novel assay for directly
viewing binding competition between QB-site inhibitors
in vivo

Introduction

The PS II complex contains integral membrane and extrinsic proteins. The D1, D2 and cytochrome b_{559} proteins constitute the core of the membrane embedded component (Nanba and Satoh, 1987; Marder et al., 1987). D2 and D1 are respectively the apoproteins of the primary (Q_A) and secondary (Q_B) plastoquinone acceptors (Rochaix et al., 1984). The PS II complex functions as a light-driven plastoquinone reductase. Inhibitors of PS II function, some of which are effective herbicides, are considered to block Q_B reduction by displacing this quinone from the Q_B -niche on the D1 protein (Velthuys, 1981; Oettmeier and Soll, 1983; Vermaas et al., 1983). Only one molecule can bind in this environment with high affinity at any given moment (Tischer and Strotmann, 1977; Laasch et al., 1982). Displacement reactions between PS II inhibitors and quinones, or between different inhibitors, can be monitored *in vitro* as a release of one radioactive compound from the thylakoid membrane upon addition of a second non-radioactive compound (Tischer and Strotmann, 1977). Quantification of these displacement reactions resulted in the widely accepted hypothesis of seemingly-competitive binding interactions (Tischer and Strotmann, 1977; Vermaas et al., 1983).

Questions concerning the specificity of the displacement assay have, however, persisted and were fuelled by the finding that large numbers of compounds are capable of competing [^{14}C] atrazine from the membrane (Gressel, 1985). For example, atrazine is displaced from isolated membranes both by other PS II inhibitors and by chemically unrelated insecticides such as DDT, dieldrin and aldrin (Moreland and Novitzky, 1984). Ethanol also competitively inhibits binding of atrazine and diuron in isolated thylakoids (Chow et al., 1990). The concentrations of ethanol used for competition, do not impede uncoupled

electron transport *in vitro* (Masamoto and Nishimura, 1978), triggering the question whether the displacement is really at the level of the Q_B niche.

The *in vivo* validity of the widely-accepted concept of seemingly-competitive binding interactions, is scarcely proven. Displacement *in vivo* could only be measured indirectly in unicellular algae as a change in the extracellular, unbound inhibitor concentration (Laasch et al., 1981, 1982). We have developed a novel, *in vivo* assay measuring competitive binding interactions directly at the Q_B niche. Our assay excludes possible isolation artifacts and examines the relevance of the concept of binding competition in the intact higher plant. Binding site competition *in vivo* is visualized, using the differential effects of PS II inhibitors on the rate of degradation of the D1 herbicide binding protein.

The D1 protein rapidly turns over with rates of synthesis and degradation exceeding those of other thylakoid proteins (Mattoo et al., 1984; Gounaris et al., 1987). The protein can be stabilized in the light by application of some, but not all PS II inhibitors (Jansen et al., 1990, 1992). We show that inhibition of D1 degradation by atrazine can be correlated to binding to the D1 protein in experiments using atrazine-resistant and sensitive *Solanum nigrum*. Atrazine, and diuron impede D1 protein degradation and photosynthetic oxygen evolution in *Spirodela oligorrhiza* with the same concentration dependence (Jansen et al., 1990, 1992). Other PS II inhibitors such as bromoxynil and bromonitrothymol (BNT) do not impair D1 degradation at concentrations almost abolishing oxygen evolution (Jansen et al., 1990; Jansen et al., in preparation). These differential inhibitor effects on D1 degradation have been related to specific inhibitor-protein interactions in the Q_B niche (Jansen et al., in preparation). Inhibition of D1 degradation thus reflects binding of compounds such as atrazine or diuron but not bromoxynil or BNT. In this

paper we visualize binding site competition between BNT and diuron by monitoring the diuron-mediated inhibition of D1 degradation in intact *Spirodela* plants.

Materials and methods

Axenic cultures of *Spirodela oligorrhiza* (Kurtz) Helgm. were grown photoautotrophically at 23°C under continuous irradiation from cool white fluorescent lamps. *In vivo* protein degradation assays were carried out as previously described (Greenberg et al., 1987; Jansen et al., 1990). Plants were radiolabeled with [³⁵S]methionine for 1 hour under 25 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light and chased either in the presence or absence of inhibitors under low light conditions (6 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ cool white fluorescent light). To stop the chase reaction, the medium was drained and the plants were kept frozen until homogenization. Membrane proteins were separated by SDS-PAGE and visualized by autoradiography (Greenberg et al., 1987). The rate of degradation of the D1 protein, relative to the stable light harvesting chlorophyll a/b protein, was quantified by microdensitometry (Greenberg et al., 1987).

Triazine-resistant and sensitive biotypes of *Solanum nigrum* (Goloubinoff et al., 1984) were grown at 24°C under a daily cycle of 16 h light of cool-white fluorescent lamps and 8 h dark. For pulse-chase experiments leaf discs were floated for 1-2 h on half strength Hutner's medium (cf. Posner, 1967) without sucrose, in the light. Following this incubation, leaf discs were transferred to medium containing 0.4% Tween 20 and 600 μCi [³⁵S]methionine. After 3 h of labeling, discs were washed with fresh medium containing 1 mM methionine. The *in vivo* chase of the labeled proteins was carried out in the presence of 1mM methionine with or without 10 μM atrazine or 10 μM diuron. The chase

reaction was stopped by freezing the leaf discs. Membrane proteins were separated by SDS-PAGE and visualized by fluorography (Reisfeld et al., 1987).

Relative rates of oxygen evolution were measured in *Spirodela*, *in vivo* by photoacoustic spectroscopy (Jansen et al., 1990). Measuring light ($15 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was modulated at a frequency of 15 Hz. Signals were processed by a lock-in amplifier (SR 530, Stanford Research systems, U.S.A.) using vectorial analysis (Poulet et al., 1983). The (inhibited) rates of oxygen evolution remained constant during the time-span of the chase experiment indicating the continuous presence of PS II inhibitors.

Binding of inhibitor to thylakoids was assayed according to Tischer and Strotmann (1977). Thylakoids (final concentration $100 \mu\text{g}$ chlorophyll/ml) were isolated from *Spirodela* fronds and incubated in medium containing 0.1 M sorbitol, 10 mM MgCl_2 , 10 mM NaCl, 10 mM tricine/NaOH (Elich et al., 1992). Freshly isolated thylakoids were incubated for 15 minutes in the dark with [^{14}C] diuron (57 mCi/mmol) in the absence or presence of BNT.

Thylakoids were spun down and the clear supernatant was mixed with a scintillation mixture of xylene and lumax (70 : 30) and counted. The difference between the amount of diuron added to the thylakoids and that remaining in the supernatant was taken as bound diuron.

RESULTS

Diuron and BNT block electron flow at the acceptor side of PS II (Trebst and Draber, 1977; Reimer et al., 1979). Binding of [14 C] diuron and BNT was studied *in vitro* using the classical herbicide displacement assay described by Tischer and Strotmann (1977). The double reciprocal presentation of the data shows that a rise in the concentration of free diuron resulted in increased diuron binding to the *Spirodela* thylakoids (Fig. 1). Extrapolation (negative inverse of intercept with X-axis) yields the dissociation constant (K_d) for diuron binding. The K_d represents the concentration of free diuron needed to occupy 50% of the available sites and had a value of 24 nM (Fig. 1). This is comparable to reported K_d values for diuron binding of 19 nM to 34 nM (Laasch et al., 1981; Oettmeier and Soll, 1983; Boschetti et al., 1985; Chow et al., 1990). Inhibitor binding is directly related to the inhibition of linear electron flow (Tischer and Strotmann 1977; Oettmeier et al., 1982). Indeed, the I_{50} value for the inhibition of electron flow in isolated thylakoids is generally quoted to range between 20 (Boschetti et al., 1985) to 40 nM (Tischer and Strotmann, 1977) diuron.

Addition of BNT to the *Spirodela* thylakoids decreased diuron binding (Fig. 1). A minor decrease in binding was observed after addition of 0.1 μ M BNT, while a major decrease was observed after addition of 1 μ M BNT. The extrapolated K_d increased 5-fold to a value of 120 nM diuron, upon addition of 1 μ M BNT (Fig. 1). Yet, the extrapolated maximal diuron binding on a per chlorophyll basis (inverse of intercept with Y-axis) was unchanged despite additions of BNT (Fig. 1). This indicates a competitive interaction between diuron and BNT, as has been reported for other pairs of PS II inhibitors (Tischer and Strotmann, 1977; Vermaas et al., 1984).

The labile D1 protein can be stabilized in the light by atrazine and diuron (Mattoo et al., 1984; Jansen et al., 1990, 1992). Using atrazine-resistant and sensitive biotypes of *Solanum nigrum* we show that the impairment of D1 protein degradation is a result of inhibitor binding to this protein. The chloroplast psbA gene of these *Solanum* biotypes was shown to be isogenic except for a single nucleotide change, which is correlated with triazine resistance (Goloubinoff et al., 1984). Both diuron and atrazine bind in the sensitive biotype (Mattoo et al., 1986), and both markedly inhibit D1 degradation (Fig. 2). Only diuron is bound in the atrazine-resistant biotype (Mattoo et al., 1986) and likewise only diuron inhibits D1 protein degradation (Fig. 2).

Binding-site competition *in vivo* was monitored in *Spirodela* as the diuron-mediated inhibition of D1 protein degradation. The rate of degradation of the D1 reaction centre protein was 0.07 h^{-1} at a low flux density of $6 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The same rate of photosynthetic oxygen evolution can be measured at all times during the pulse-chase experiment. Thus, there is an apparent balance between synthesis and degradation. The addition of $0.3 \mu\text{M}$ diuron resulted in approximately equal decreases in the rates of oxygen evolution and D1 degradation (Fig. 3). A concentration of $1 \mu\text{M}$ BNT under the conditions used, only slightly affected D1 degradation although photosynthetic electron flow is almost abolished (Fig. 3). Thus, inhibition of D1 degradation almost exclusively reflects the binding of diuron, but not BNT. Exposure of plants to a mixture of $1 \mu\text{M}$ BNT and $0.3 \mu\text{M}$ diuron resulted in a significantly higher rate of D1 degradation than in the presence of $0.3 \mu\text{M}$ diuron alone, while photosynthetic electron flow was more severely blocked (Fig. 3). We interpret this to reflect decreased diuron binding due to competition by BNT in the first instance, and the increase in the total concentration of PS II inhibitors in the second.

The addition of 1 μM of BNT resulted in an increase in the D1 degradation rate at all diuron concentrations used (Fig. 4). The I_{50} concentration for the inhibition of D1 degradation increased 10-fold from 0.3 μM to 3 μM diuron upon addition of 1 μM BNT (Fig. 4).

Discussion

Diuron binding was monitored *in vivo*, as the inhibition of D1 protein degradation. Displacement of diuron by BNT was monitored as a reduction in the diuron-mediated inhibition of D1 degradation. Both *in vitro* (Fig. 1), and *in vivo* (Figs. 3, 4) addition of BNT resulted in decreased diuron binding. Increasing the diuron concentration nullified the effect of added BNT both *in vitro* (Fig. 1) and *in vivo* (Fig. 3, 4). Thus, strong similarities exist between binding-site competition observed *in vitro* and *in vivo*. Our results demonstrate the validity of the *in vitro* derived concept of herbicide-displacement in the intact higher plant.

A quantitative comparison between binding-site competition *in vivo* and *in vitro* (Fig. 1, 4), shows that slightly more diuron is needed to overcome BNT binding *in vivo* (10-fold) than *in vitro* (5-fold). The comparison is fair as effective BNT concentrations *in vitro* ($I_{50} = 0.2 \mu\text{M}$) are very similar to those *in vivo* in *Spirodela* ($I_{50} = 0.3 \mu\text{M}$)(Jansen in preparation). *In vitro* binding of PS II inhibitors depends on a number of physiological parameters such as stacking of the membranes (Vermaas et al., 1984), bicarbonate binding to PS II (Vermaas et al., 84), protein phosphorylation (Shochat et al., 1982), the presence of light (Vermaas et al., 1984) and intactness of the quinone and herbicide binding environment (Chow et al., 1990). Moreover, these parameters affect binding of PS II inhibitors in a compound specific manner (Vermaas et al.,

1984). Still, *in vitro* and *in vivo* data on competition between diuron and BNT are quantitatively similar. We conclude from this that the *in vivo* and *in vitro* inhibitor binding environments are highly similar.

The traditional thylakoid inhibitor binding assay (Tischer and Strotmann, 1977) measures binding as a decrease in the quantity of non-bound inhibitor. Only a small portion of the inhibitor is bound. Furthermore, non-specific binding may be of significance. Both factors are problematic and limit *in vivo* experiments to unicellular algae (Laasch et al., 1981, 1982). The non-specific adsorption of inhibitors does not impair D1 degradation, as can be seen from the lack of effects of atrazine on a triazine-resistant biotype (Fig. 2). Thus, this artifact is not monitored in the D1 degradation assay. Conversely, the *in vivo* assay is limited to pairs of PS II inhibitors that differently effect D1 degradation. Still, the D1 degradation assay is preferable under *in vivo* conditions, as it visualizes inhibitor binding directly at the level of the Q_B niche.

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Legends

Fig. 1 Lineweaver-Burk plot showing binding of [^{14}C] diuron in *Spirodela* thylakoid membranes. Control ($\square$), $R^2 = 0.991$, $K_d = 24$ nM; +0.1 μM BNT ($\bullet$), $R^2 = 0.939$, $K_d = 26$ nM; +1.0 μM BNT ($\blacktriangle$), $R^2 = 0.995$, $K_d = 112$ nM.

Fig. 2 Degradation of the D1 protein in triazine-resistant and -sensitive biotypes of *Solanum nigrum* in the absence (untreated) and presence of 10 μM atrazine (+atrazine) or 10 μM diuron (+diuron). Samples were loaded on SDS-polyacrylamide gels on an equal radioactivity basis. Fluorographs are shown. The position of the D1 protein is indicated.

Fig. 3 Inhibition of D1 degradation and photosynthetic oxygen evolution by BNT and/or diuron. Rates in the absence of any inhibitor were normalized to 100% (rate of D1 degradation 0.07 h^{-1}). Standard errors are shown.

Fig. 4 Inhibition of D1 degradation by diuron in the presence or absence of 1 μM BNT. Rates in the absence of diuron were normalized to 100% (no inhibitor, $0.068 \pm 0.005\text{ h}^{-1}$; +1 μM BNT, $0.057 \pm 0.007\text{ h}^{-1}$). Standard errors are shown.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Chapter 4. The D1-D2 heterodimer of PS II is a major target for
UV-B irradiation

Introduction

Stratospheric ozone depletion due to man-made pollution is expected to lead to more UV-B penetration to the biosphere (Crutzen, 1992) and negatively impact crop productivity and quality. Plant exposure to increased UV-B levels leads to alterations in growth and development (Tevini et al. 1981), transpiration (Tevini and Teramura 1989) and photosynthesis (Brandle et al. 1977, Bornman 1989). Not all plant species are sensitive, (Teramura 1983) and considerable variation in response exists also intraspecifically (Murali and Teramura 1986a, Murali et al. 1988). Most plants accumulate UV absorbing pigments in their leaf epidermis as a protective measure following exposure to UV-B radiation (Robberecht and Caldwell 1983). Evidence has accumulated showing relatively greater increases in flavonoids in UV-B resistant species following irradiation (cf. Tevini and Teramura 1989). It is not known to which extent UV absorbing pigments can protect against increasing levels of UV irradiation. To combat potential damage to crops it is important to identify and monitor key cellular targets sensitive to UV-B-damage.

Photosynthesis is one of the plant systems directly damaged by UV-B radiation (Bornman 1989). Experimental evidence points to photosystem II (PS II) as the main receptacle for UV-B damage in photosynthesis (Brandle et al. 1977, Noorudeen and Kulandaivelu 1982, Iwanzik et al. 1983, Tevini and Pfister 1985, David et al. 1988,

Renger et al. 1989). The PS II multimolecular protein-pigment complex consists of a water splitting system, a light harvesting chlorophyll protein complex and a thylakoid embedded reaction centre (Rochaix and Erickson 1988, Mattoo et al. 1989). The D2 and D1 proteins form the core of the PS II reaction centre (Deisenhofer et al. 1985). Both proteins, together with two cytochrome b559 subunits, bind several chlorophyll, pheophytin, and carotene molecules as well as non-haem iron (Nanba and Satoh 1987, Marder et al. 1987a). D2 and D1 are, respectively, the apoproteins of the primary quinone electron acceptor, Q_A , and the secondary quinone acceptor, Q_B . The D1 protein mediates electron flow to the plastoquinone pool by binding and unbinding Q_B in various redox states (Crofts and Wraight 1983).

Several studies reported the water splitting system to be a major target of UV-B radiation stress within PS II (see Bornman 1989). Electron transport measurements were performed on isolated PS II membrane preparations before and after UV-B irradiation. These indicated a deterioration specifically in the function of water oxidation (Renger et al. 1989). However, the 33 kDa oxygen-evolving-complex protein was not significantly affected. Rather, it was concluded that structural changes in the D1-D2 polypeptide matrix were responsible for the UV-B induced effects (Renger et al. 1989).

The D1-protein is a major product of the chloroplast protein synthesizing machinery (Edelman and Reisfeld 1979). However due to its labile character only a low steady state amount is present. Turnover is driven by visible (Mattoo et al. 1984), far-red (Gaba et al. 1987) and UV (Greenberg et al. 1989) radiation. The homologous D2 protein is stable under photosynthetic active radiation (Mattoo et al. 1984). Yet, we demonstrate UV-B driven degradation of the D2 protein. We present data showing that the D1-D2 heterodimer of PS II is a major target for UV-B type damage.

Materials and Methods

All experiments are performed *in vivo* with the aquatic higher plant *Spirodela oligorrhiza* as described by Gaba et al. (1987). *Spirodela* was grown photoautotrophically under $10 \mu\text{E.m}^{-2}.\text{s}^{-1}$ photosynthetic active radiation (PAR) provided by cool white fluorescent lamps. Fronds were pulse labeled with $[^{35}\text{S}]$ methionine for 1 hour under $25 \mu\text{E.m}^{-2}.\text{s}^{-1}$ photosynthetic active radiation. Chase conditions are detailed in the legends. Membrane proteins were fractionated by SDS-PAGE and visualized by autoradiography. Degradation of proteins was quantified by densitometry (Marder et al. 1987b).

Radiation sources are given in the hierarchy: wavelength (half-power band width); 254 nm (1 nm Hg emission line), 300 nm (40 nm),

and 350 nm (30 nm); Rayonet photoreactor bulbs, 280 nm (30 nm); 900-W Xe lamp with monochromator (Schoeffel), 313 nm, 366 nm, and 405 nm (1 nm, Hg emission lines); 250-W Hg lamp fitted with GG19+UG11 (Schott), UG1+WG360 (Schott), and GG385 (Schott)+7-59 (Corning) filters, respectively; 429 nm, 447 nm, 560 nm, 660 nm, 716 nm, and 731 nm (all 10-20 nm); 250-W tungsten halogen projectors fitted with a heat absorbing glass plus the appropriate interference filter (Schott, Balzers, or Dittic). Visible light was generated with cool-white fluorescent bulbs ($75 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or with a 250-W Tungsten halogen projector with a heat absorbing glass and a 1.5 cm waterfilter ($1000 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Photon fluence rates were measured with a LiCor quantum flux meter (visible and far-red) or a potassium ferrioxalate actinometer (UV).

PS II inhibitors were obtained from Riedel-de Haën AG, F.R.G. (diuron, atrazine), or Chem Service, P.A., U.S.A. (DNOC, dinoseb, bromoxynil, ioxynil). Pyridate was a kind gift of Chemie Linz AG, Austria. Substituted nitrophenols (BNT, 6-cyclic hexyl bromonitrophenol) were kindly provided by Dr. W. Draber, Forschungszentrum Bayer AG, F.R.G.. The application of PS II inhibitors, and the *in vivo* measurements of photosynthetic oxygen evolution using photoacoustic spectroscopy, have been detailed previously (Jansen *et al.* 1990).

Results

Degradation of the PS II reaction centre proteins D1 and D2, can be visualized as the disappearance of the labeled protein band in autoradiograms. D1 degrades in UV-B and red radiation with similar kinetics, but is stable in the dark (Fig. 1). The metabolism of the D2 protein was also studied (Fig. 1). D2 is relatively stable in the dark and in red radiation, but its rate of degradation increases by more than an order of magnitude if exposed to UV-B radiation. Under this latter condition, both D1 and D2 may be considered as rapidly turning over proteins (Fig. 1). We note that rates of UV-B driven degradation vary somewhat from one set of experiments to another. This may be related to differences between plants in chlorophyll content (Greenberg et al., 1989) or UV-B shielding pigments.

The rates of degradation of the D1 and D2 proteins under UV-B radiation increase with increasing flux densities, seemingly reaching a plateau by $80 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Fig. 2). The UV-B driven degradation of the D1 and D2 proteins is specific as other PS II proteins, such as the chlorophyll binding PS II core proteins CP43, CP47, and the proteins of the light-harvesting chlorophyll a/b complex (LHCII), are stable under these conditions. Likewise, the large subunit (LS) of RUBISCO is stable (Fig. 2).

Mixed light experiments, designed to approximate outdoor conditions, show that a photosynthesis saturating flux density of ($1000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) PAR reacts synergistically with low levels of UV-B radiation to yield a highly elevated rate of D2 degradation (Fig. 3). D1 degradation is less strongly stimulated by light mixing (Fig. 3). As a consequence, the rate of D2 degradation approaches the high rate of D1 degradation under this condition. The synergistic stimulation of the rates of D1 and D2 degradation, observed after mixing UV-B radiation with a background of visible light, is UV-B flux density dependent (Fig. 3). No synergistic UV-B effect is discernable below a threshold intensity. The enhancement of the visible light effect, relative to the effect of UV-B only, shows an optimum at about $1 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Fig. 3). This flux density triggers a strong synergistic response if combined with PAR, but causes by itself only minor degradation of the D1 and D2 proteins. At $6 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ UV-B, the synergistic increase in the degradation rates has become relatively small as compared to the effects of pure UV-B.

The synergistic effect on D2 protein degradation (Fig. 3) is UV-B specific; UV-A does not cause a synergistic stimulation of either D2 or D1 degradation in combination with visible light (data not shown).

In vivo action spectra for degradation of D1 and D2 have been determined. Spectra in the UV region (254-366 nm) for D1 and D2 degradation have comparable shapes (Fig. 4), with a peak at 313

nm, suggesting similar photosensitizers. The resemblance of the D1 degradation spectrum with the absorption spectrum of plastosemiquinones has been noticed previously (Greenberg et al., 1989). We surmised that if semiquinones act as photosensitizers in the UV-B, any PS II inhibitor which displaces Q_B from its site on D1, should impair UV-B driven D1 degradation. PS II inhibitor binding *in vivo* was measured under PAR as a decrease in photosynthetic oxygen evolution. In parallel, we measured inhibitor effects on D1 degradation in plants exposed to either visible or UV-B radiation. All PS II inhibitors tested, impair D1 degradation in UV-B to roughly the same extent as oxygen evolution under PAR (Table 1). The impairment of UV-B driven degradation is accentuated by noting that several of the tested PS II inhibitors do not inhibit degradation of the D1 protein in visible light (Table 1). Thus the presence of Q_B in its site on the D1 protein is a specific requirement for UV-B driven D1 degradation. The differential inhibitor effects on visible light-driven D1 degradation have been related to specific inhibitor-protein interactions in the Q_B-niche (Jansen et al., in preparation).

Discussion

The D1 reaction centre protein rapidly turns over if exposed to photosynthetic active radiation (Mattoo et al. 1984). Under such light conditions the homologous D2 protein is stable (Mattoo et al. 1984). We observed high rates of degradation of both the D1 and the

D2 protein when plants are exposed to UV-B radiation (Fig. 1,2,4). Rates of D1 degradation are only slightly higher in UV-B as compared to visible light. Other chloroplast localized proteins, like CP43, CP47, LHCII and the LS of RUBISCO were found to be stable under both light environments (Fig. 2). Thus, the D1-D2 heterodimer is a specific target for UV-B radiation. Degradation of the D1 protein is well documented, and schemes for a repair cycle involving reaction centre trafficking from grana to stroma lamellae have been proposed (Callahan et al. 1989). Presumably plants are well prepared to cope with D1 turnover since exposure to photosynthetically non-saturating intensities of visible light does normally not lead to a decrease in photosynthetic performance. Under conditions of enhanced UV-B radiation, the D1-D2 core of the PS II reaction centre is destroyed and presumably the whole PS II centre disassembles. This results in a considerable cost of energy for resynthesis and reassembly of a new reaction centre. Concomitantly, a decrease in photosynthetic performance can be monitored (Brandle et al. 1977, Noorudeen and Kulandaivelu 1982, Iwanzik et al. 1983, Tevini and Pfister 1985, David et al. 1988, Renger et al. 1989). Indeed, the *in vivo* D1 and D2 degradation spectra, resemble roughly the *in vitro* spectra for UV inhibition of PS II electron flow (Renger et al. 1989, Bornman et al. 1984). Deviations between the spectra in the UV-C range suggest that mechanisms other than destruction of the D1-D2 heterodimer may play a role in PS II inactivation at these non-physiological wavelengths.

UV-B driven degradation of the D1-D2 heterodimer is of potential significance, due to the increase in solar UV-B penetration in the biosphere. Sunlight reaching the earth's surface consists of about 0.25% of radiation with a wavelength shorter than 330 nm (Gaba, unpublished). These wavelengths (290-330 nm) could on a sunny day constitute about 4- to 6 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. We mixed a small amount of UV with several hundred-fold more visible radiation to mimic exposure to sun light. This results in disproportionately strongly elevated rates of degradation of D2 and to a lesser extent D1 (fig. 3). We show that any increase in UV-B radiation further decreases the stability of the D1 and D2 proteins. Yet, it depends on the UV-B flux density whether the increase in UV-B radiation will cause a synergistic or merely an additive destabilisation of the D1-D2 heterodimer (Fig. 3). Most "field adapted" plants are able to accumulate UV-absorbing pigments (Robberecht and Caldwell 1983) thus avoiding harmful UV-B radiation reaching their chloroplasts. We surmise that plants are normally able to reduce UV-B penetration such that the extent of destabilization of the D1-D2 heterodimer is limited (e.g. below the threshold in figure 3). Therefore, any increase in the flux density of UV-B, may trigger a synergistic destabilization of the D1-D2 heterodimer (Fig. 3), unless matched by a sufficient increase in UV-B shielding capacity. The outcome of this race between increased UV-B radiation and increased UV-B shielding capacity seems to be species (Teramura, 1983) and cultivar dependent (Murali and Teramura 1986a, Murali

et al., 1988). We predict that by measuring the degradation rates of both D1 and D2, UV-B penetration in plants can be assessed.

The photosensitizer(s) for degradation of the D1 and D2 proteins are similar compounds as judged from the similar *in vivo* degradation spectra (Fig. 4). The resemblance between the D1 degradation spectrum and the absorption spectrum of plastosemiquinones has been noticed (Greenberg et al., 1989). The possibility that UV-B radiation excites the quinone bound at the Q_B-site and subsequently affects the stability of the D1 protein was considered (Greenberg et al., 1989). Yet the question was raised why UV-B radiation does not excite the quinone bound at the Q_A-site and cause also degradation of the D2 protein (Ohad review). We have now shown for the first time that D2 degradation is strongly stimulated in UV-B light, thus supporting the hypothesis that semiquinones act as photosensitizers. This hypothesis may also explain the synergistic stimulation of degradation of D1 and D2, observed in light mixing experiments (Fig. 3). Semiquinones are formed transiently during photosynthetic electron flow. Thus, visible light poises Q_A and Q_B in the redox state which does absorb UV-B radiation. The stabilization of the D1 protein in UV-B by PS II inhibitors which displace Q_B (Table 1), strongly supports the hypothesis of semiquinones acting as photosensitizers in UV-B driven D1 degradation. Previously, tyrosine radicals have also been hypothesised as photosensitizers for UV-B driven degradation. D1 and D2 carry respectively the redox active tyrosines Z and D.

However, the tyrosine radical spectrum differs from the D1 degradation spectrum in having a major peak close to 254 nm (ref). Furthermore, the PS II inhibitors dinoseb and diuron, which are both thought to displace Q_B and which similarly inhibit D1 and D2 degradation, have strikingly different effects on the EPR signal from the oxidized radical Tyr 160 at D2 (ref). EPR signal II is abolished by dinoseb, yet diuron does not affect its steady state amplitude.

The inhibition of D2 degradation (Table 1) is analogous to the inhibition of D2 trypsinization by PS II inhibitors in the Q_B niche (Trebst et al., 1988). This may indicate that the D2 protein is stabilised by the D1 protein and can only degrade in its absence. Indeed, rates of D2 degradation approach, but never exceed rates of D1 degradation. The close physical interaction between stretches of residues from the quinone/inhibitor binding region of D1 with stretches of residues of the analogous region of D2 (Trebst, 1997/Kless D2 paper) may facilitate the stabilisation of D2 by D1.

In summary, the data presented identify the D1-D2 heterodimer of photosystem II as a major, specific target of UV-B radiation.

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Inhibitor	Light Conditions			
	Visible		UV-B	
	% Oxygen evolution	% D1 degrad.	% D1 degrad.	% D2 degrad.
Diuron	50	56	46 ± 8	37 ± 11
BNT	29	99	34 ± 6	22 ± 14
cycl. hexyl BNT	36	29	32 ± 10	31 ± 7
Bromoxynil	27	93	30 ± 3	55 ± 10
loxynil	38	41	32 ± 6	58 ± 9
Pyridate	31	76	35 ± 5	77 ± 10
DNOC	12	66	29 ± 9	26 ± 15
Dinoseb	60	40	43 ± 4	39 ± 6

Table 1

Legends

Figure 1. Degradation of the D1 and D2 proteins in red and UV-B light. Amounts of protein were assessed at the times indicated. Flux densities were $10.5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. S.E. is shown.

Figure 2. Degradation rates of the D1 and D2 reaction centre proteins, the chlorophyll binding proteins CP43 and CP47 and the large subunit of RUBISCO (LS) under UV-B radiation. The straight baseline represents LHCII, to which all other data are normalized. S.E. is shown.

Figure 3. Degradation rates of the D1 and D2 proteins in mixtures of white and UV-B light. The effect of UV-B is given either in the absence () or in the presence () of the background white light intensity of $1000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. S.E. is shown.

Figure 4. *In vivo* action spectra for degradation of the D1 and D2 proteins. The rate of degradation for equal quanta of incident radiation ($6 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) is given. S.E. is shown.

Fig. 1

Fig. 2

Fig. 3

UV-B radiation ($\mu\text{E}/\text{m}^2\text{s}$)

Fig. 4

Wavelength (nm)

Chapter 5 A PS II reaction center particle from stromal lamellae

Photosynthetic electron transport from H_2O to $NADP^+$ requires the concerted action of three protein complexes: Photosystem II (PSII), the cytochrome b_6/f complex (cytb₆/f) and Photosystem I (PSI). Photosystem I complexes, concentrated in the unappressed stroma lamellae (Callahan et al., 1989), are mostly homogeneous in protein, pigment composition, and electron transport properties (Armond & Arntzen, 1977; Haehnel, 1984; Anderson, 1986; Melis, 1991), although some heterogeneity might exist (Andreasson et al., 1988 and Svensson et al., 1991). PSII complexes, on the other hand, are heterogeneous both in chlorophyll (Chl) antenna size (Armond & Arntzen, 1977; Thielen & Van Gorkom, 1981; Melis & Homann, 1976), and their capability to reduce the plastoquinone (PQ) pool (Lavergne, 1982; Melis, 1984; Graan and Ort, 1986; Chylla et al., 1987). The functional heterogeneity of PSII complexes has been correlated with their lateral distribution in mature higher plant thylakoids. PSII complexes with smaller antennae and with impaired PQ-reducing properties are present in the unappressed stroma lamellae, while PSII complexes containing larger antennae and capable of reducing PQ are concentrated in the appressed (grana) lamellae (Anderson and Melis, 1983; Ghirardi et al., 1986; Albertsson and Shi-Gui, 1988; Henrysson and Sundby, 1990).

Photosystem II complexes consist of a reaction center (D1, D2, the α and β subunits of cytochrome b_{559} , and the PsbI protein), a peripheral oxygen-evolving complex, a set of ancillary Chl a -containing antenna polypeptides, and a Chl a/b -containing light-harvesting antenna. Most of these components are present in both the appressed (grana) and unappressed (stroma lamellae) membranes (Vallon et al., 1986; Callahan et al., 1989). The identification of the D1 protein as a component of an active PSII reaction center (Nanba & Satoh, 1987) has raised

a physiological dilemma due to the fact that D1 undergoes a rapid turnover in the light which is several times higher than other thylakoid components (Eaglesham and Ellis, 1974; Reisfeld and Edelman, 1978; Grebanier et al., 1979; Mattoo et al., 1984; Edelman et al., 1984). The light-dependent turnover of D1 implies that PSII reaction centers are constantly undergoing assembly/disassembly in the membrane. Moreover, the D1 protein is known to be synthesized and processed to its mature form in the stroma lamellae (Mattoo and Edelman, 1987); however, most of the protein is found associated with the PSII reaction center in the grana (Callahan et al., 1989). These data have led to speculation that D1, and possibly the entire reaction center, cycles between the two membrane regions.

Grana-localized intact PSII reaction center particles have been isolated from a number of different organisms and extensively characterized with respect to their composition and functional capability in light absorption and utilization for charge separation (Nanba and Satoh, 1987; Barber et al., 1987; Seibert et al., 1988; Shuvalov et al., 1989; Tetenkin et al., 1989; Braun et al., 1990). However, a fully assembled stroma lamellar PSII reaction center has not been isolated or identified. We report here the isolation of an assembled PSII reaction center from stroma lamellae and present its pigment and polypeptide composition, and characteristics of excitation transfer from different pigments to P_{680} .

EXPERIMENTAL PROCEDURES

Isolation of PSII reaction centers - *Spirodela oligorrhiza* plants were grown in sucrose-supplemented medium under $20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ cool fluorescent light at 20-25°C. Thylakoids were isolated as described (Marder et al., 1986) and fractionated into grana and stroma lamellae by the dual detergent method (Callahan et al., 1989). Thylakoid fractions were resuspended in 50 mM Tris-NaOH, pH 7.2, and washed once with 50 mM Tris-NaOH, pH 9.0, to remove peripheral proteins. Grana and stroma lamellar PSII reaction centers were isolated from pH 9.0 buffer-washed membranes as described for grana (Nanba & Satoh, 1987): five mg Chl equivalent of grana or 15 mg Chl equivalent of stroma lamellae at a concentration of 1 mg Chl/ml were solubilized in 4% Triton X-100 and incubated at 4°C for 1 h while stirring. The insoluble material was removed by centrifugation at $100,000 \times g$ for 1 h and the supernatant loaded onto a 4-cm long DEAE-650S Toyopearl column (Supelco; Rohm & Haas). All procedures were carried out at 4°C under dim lights. The column was washed extensively with 50 mM Tris-NaOH, pH 7.2, containing 30 mM NaCl and 0.05% Triton X-100. The reaction centers were eluted with a 30-400 mM NaCl gradient. Reaction centers from either membrane fraction eluted at about 100 mM NaCl. The reaction center fractions were combined, diluted three times with 50 mM Tris-NaOH, pH 7.2, in the presence of 0.05% Triton X-100 and loaded onto a 1-cm DEAE-Toyopearl column. The second column was washed with 5 ml of 50 mM Tris-NaOH, pH 7.2, containing 30 mM NaCl and 2 mM dodecyl maltoside. The bound reaction centers were eluted with 200 mM NaCl in the same buffer (McTavish et al., 1989).

Polypeptide composition - The polypeptide composition of the isolated reaction center fractions was analyzed by SDS-PAGE using 10-20% gradient polyacrylamide gels (Marder et al., 1986). Following electrophoretic separation, proteins were either stained with silver or electroblotted onto nitrocellulose paper and probed with different antibodies (Callahan et al., 1989). Immunodetection was done by the alkaline phosphatase-linked secondary antibody method. The relative concentration of proteins in the blots was determined from densitometric scans done with an LKB laser densitometer.

Spectroscopic characterization - The absorption spectrum of the reaction center preparations was done at room temperature with a DU-70 Beckman spectrophotometer while 77K fluorescence emission and excitation spectra were obtained with a SLM 8000 spectrofluorometer.

RESULTS

We previously used the dual-detergent method of Leto et al. (1985) to fractionate thylakoids from *Spirodela* into highly resolved grana and stroma-lamellae (Callahan et al., 1989). Immunocytochemical analysis revealed less than 2% contamination of one type of membrane fraction with the other. Both fractions were treated with Triton X-100 at the same Triton:Chl ratio as described by Nanba and Satoh (1987), loaded onto a DEAE-650S Toyopearl column, and reaction centers eluted with a NaCl gradient. The fractions enriched in PSII reaction centers were combined and reloaded onto a second, shorter column to exchange Triton X-100 with dodecyl maltoside which stabilized the subsequently eluted preparation.

Polypeptide composition of the isolated reaction centers. Reaction center fractions (20 μ g Chl/ml) from both membranes were solubilized in SDS and analyzed by SDS-PAGE. Figure 1 shows the results of the purification stages using silver-stained gels (A) and immunoblots probed with anti-D1 antibody (B). The granal reaction center preparation contained four characteristic protein bands: the 9-kDa α -subunit of cytochrome b_{559} , the 30-kDa D2, the 32-kDa D1 and a 55-kDa aggregate (Nanba and Satoh, 1987; Marder et al., 1988) of D1/D2. In our gel system neither the 5-kDa subunit of cytochrome b_{559} nor the 4.5-kDa PsbI protein (Webber et al., 1989) are retained. The identity of the protein bands was confirmed by immunoblots using anti-D1 (Fig. 1B), anti-D2 and anti-cytochrome b_{559} antibodies (not shown).

The stroma lamellar PSII reaction center fractions were enriched in the same four characteristic protein bands present in the grana reaction center fraction, however, minor protein bands at 19, 23, 47 and 43 kDa were also present. None of these latter cross-reacted with antibodies against either the D1 protein (Fig. 1B), D2 protein, cytochrome b_{559} , the 19-kDa subunit of PSI, or the 47/43-kDa PSII polypeptides (data not shown). Due to their minor accumulation, we conclude that they are contaminants or loosely-associated components that cofractionate with stroma lamellae reaction center.

The stoichiometry of the protein components of the stromal PSII reaction center was compared to that of the granal reaction centers. Purified preparations of grana and stroma lamellae reaction center were serially diluted, the polypeptides resolved by SDS-PAGE and immunoblots probed sequentially with anti-D1, anti-D2 and anti-cytochrome b_{559} antibodies. The resulting blots were scanned and the relative amount of each immuno-reactive band was determined.

We normalized all values to the amount of Chl in each lane and averaged the results, as shown in Table I. Grana and stroma lamellae reaction center preparations contained approximately the same relative amount of D1 and D2 per Chl, indicative of 1:1 stoichiometry. The relative amount of cytochrome b_{559} per Chl in stroma lamellar reaction centers, however, was about 60% of that in grana preparations (Table I). This could reflect: (a) the actual composition of stroma lamellar PSII reaction centers, in agreement with Vallon et al. (1987); (b) a weak association of cytochrome b_{559} with the D1/D2 heterodimer in stroma lamellar PSII reaction center, resulting in its selective loss during the purification procedure; or (c) a selective depletion of cytochrome b_{559} from stroma lamellar reaction center preparations due to the higher Triton/PSII ratio to which these membranes were submitted during the isolation procedure, in agreement with Tang et al. (1990).

Biophysical characteristics of the isolated reaction centers. Stroma-lamellae-located PSII complexes differ somewhat from the granal complexes. The stroma lamellae complexes have smaller Chl antennae cross section, are not able to transfer electrons as efficiently to the PQ pool, and were reported to have lower efficiency of charge separation (Holzworth et al., 1985). These differences have been attributed, respectively, to: (a) lack of an associated peripheral light-harvesting antenna (Anderson and Melis, 1983); (b) absence of significant amounts of PQ in the membrane domain occupied by PSII complexes in stroma lamellae (Melis, 1985); and (c) inherent differences in the reaction center properties of stroma lamellae and granal PSII centers. In order to determine which of the above hypotheses is correct, we determined several

functional properties of the isolated stroma lamellar reaction center particle.

The chromophore composition of the two reaction center preparations was determined from their room temperature absorption spectra (Figure 2). Both preparations show characteristic peaks at 417 nm (due to pheophytin and/or cytochrome b_{559}), 431.5 nm (due to the Soret transitions of Chl a), shoulders at 467 and 484 nm (due to carotenoids), a broad peak at 540 nm (due to pheophytin), and peaks at 620 and 674 nm (due to Chl a). The spectra were normalized with respect to the 674 nm Chl a absorbance peak. The table in Figure 2 shows that the relative absorbance of stroma lamellae and grana reaction centers is the same at all wavelengths and agrees well with previously published room temperature spectra for grana reaction centers (Nanba and Satoh, 1987; Barber et al., 1987; McTavish et al., 1989). These results suggest that the two types of reaction center preparations bind the same chromophores and each contains fully functional Chl a molecules, as evidenced by the peak at 674 nm (Seibert et al., 1988).

The competence of the two reaction center preparations in transferring the absorbed light to functional P680 was determined from the 77K fluorescence emission spectrum. Samples were excited with 430 nm light and Chl fluorescence was detected from 650 to 750 nm. Figure 3 shows the 77K emission spectra of grana and stroma lamellae reaction centers. Both samples exhibit a single fluorescence emission peak centered at 686 nm. Previous reports have described a shoulder at 670 nm, which has been attributed to a population of non-functional reaction centers (Shuvalov et al., 1989, Tetenkin et al., 1989). We did not observe such a shoulder in our preparations, suggesting that they are fully functional. Neither a broad peak at 730 nm (Barber et al., 1987) nor a small shoulder at 692 nm was observed (Tetenkin et al., 1989).

The relative efficiency of different pigments in transferring excitation to P680 was probed at 77 K by measuring fluorescence emission at 730 nm upon excitation of the sample with light from 400 to 700 nm. Both grana and stroma lamellae reaction center preparations showed similar excitation spectra (Fig. 4). These results confirm the fact that both reaction centers bind the same chromophores and establish that the chromophores transfer energy to P680 with the same efficiency in both preparations.

DISCUSSION

We are not aware of a prior report on the isolation of a PSII reaction center particle from stroma lamellae. Our results on the biochemical and biophysical properties of the stroma lamellae reaction center particles, including the unit stoichiometry of 1:1 of the D1 and D2 proteins in both reaction centers, show its similarity to previously-characterized granal reaction center preparations in terms of polypeptide and pigment composition, and their ability to absorb excitation energy and transfer it to P680. The isolation of a fully functional reaction center from stroma lamellae should enable addressing questions related to differences in the primary photochemistry in these reaction centers, such as quantum yields, rate constants and stability of intermediate states, and testing whether these potential differences are the source of the functional differences between stroma lamellae and granal PSII complexes.

The presence of a discrete PSII reaction center in stroma lamellae has interesting physiological implications. For instance, the D1 protein is synthesized and processed on the unappressed stroma lamellae following which it translocates to grana lamellae (Mattoo and Edelman, 1987; Adir et al., 1990).

An assembled PSII reaction center in stroma lamellae could mean that the whole PSII reaction center assembles on the stroma lamellae and then translocates to grana as a unit. This suggestion is consistent with the dual membrane location of PSII complexes (Vallon et al., 1986; Callahan et al., 1989), a dynamic phenomenon regulated by the developmental stage of the chloroplasts (Thielen and Van Gorkom, 1981; Ghirardi and Melis, 1988; Greene et al., 1988) as well as by physiological factors such as heat (Sundby et al., 1986), light intensity (Guenther and Melis, 1990; Guenther et al., 1990; Neale and Melis, 1990) and the oxidative state of the PQ pool (Kyle et al., 1982; Deng and Melis, 1986; Larsson et al., 1987).

It has been speculated that the grana-localized PSII reaction center cycles back to the stroma lamellae to replace D1 'damaged' during its stay in the grana (Ghirardi et al., 1989; Hundal et al., 1990; Adir et al., 1990). In our present study we have not detected a hypothetical D1-less PSII reaction center. However, this could be due to one of several reasons, including a short life-span of the D1-less state. The current evidence supporting movement of PSII complexes from grana to stroma lamellae is indirect: the 23.5-kDa degradation product of D1 has been detected preferentially in stroma lamellae (Ghirardi et al., 1989; Barbato et al., 1991); and the steady-state level of the PSII polypeptides, D2, cytochrome b_{559} , CP43 and CP47 increases in stroma lamellae at the cost of that in the grana fractions of spinach thylakoids submitted to photoinhibitory treatment *in vitro* (Hundal et al., 1990). The latter results could be interpreted to mean that D1 degradation occurs in the grana without involving reaction center cycling. Clearly, pulse-chase and double-labeling experiments need to be performed and distribution of the label analyzed.

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Table I

Relative amounts and ratio of the reaction center proteins D1, D2 and cytochrome b_{559} in grana and stroma lamellar PSII reaction centers.

Purified reaction centers (PSII RC) were sequentially diluted, treated with SDS and fractionated by SDS-PAGE. The resolved polypeptides were blotted onto nitrocellulose and probed with anti-D1, anti-D2 and anti-cytochrome b_{559} antibodies. The relative amounts of each protein were determined as described in the text. Each value given is the average of 5 to 12 determinations $\pm$ standard deviation.

Reaction center protein	Grana PSII RC	Stroma lamellar PSII RC	Stroma lamellar/Grana PSII RC
D1	71.8 $\pm$ 10.1	75.1 $\pm$ 11.9	1.05 $\pm$ 0.22
D2	22.5 $\pm$ 4.2	27.6 $\pm$ 5.5	1.23 $\pm$ 0.34
Cyt b_{559}	49.1 $\pm$ 11.2	30.7 $\pm$ 5.7	0.62 $\pm$ 0.18

Legends to Figures

Figure 1 - Protein composition of the isolated PSII reaction centers from grana and stroma lamellae of *Spirodela*. Solubilized grana (upper panels, lanes 1) and stroma lamellae (lower panels, lanes 1) were fractionated and processed to obtain respective PSII reaction centers, which were then analyzed on SDS-PAGE. Silver-stained gels (A) and anti-D1 immunoblots (B) of the PSII reaction center preparations are shown. Lanes 1, preparation loaded on first column; lanes 2, peak reaction center fractions from first column in 0.05% Triton; lanes 3, peak reaction center fractions from second column in 2mM dodecyl maltoside. The locations of D1, D2, cytochrome b_{559} , and the D1/D2 aggregate (which cross reacted with anti-D1 and anti-D2 antibodies) are indicated.

Figure 2 - Chromophore composition of the isolated granal and stromal lamellar PSII reaction centers. Room temperature absorption spectra of *Spirodela* granal (GL) and stroma lamellar (SL) PSII reaction centers in 2mM dodecyl maltoside were determined. Inset: relative height of each absorption peak in the two preparations.

Figure 3 - 77 K fluorescence emission spectra of granal (GL) and stroma lamellar (SL) PSII reaction centers in 2mM dodecyl maltoside, excited with 430 nm light. Peaks are centered at 686 nm. Both preparations show only one emission peak (686 nm) which is characteristic of P_{680} , the reaction center Chlorophyll of PSII. The data indicate that all the Chlorophyll molecules present in the two preparations transfer energy to P_{680} .

Figure 4 - 77 K fluorescence excitation spectra of granal (GL) and stroma lamellar (SL) PSII reaction centers in 2mM dodecyl maltoside. The samples were excited with light from 400 to 700 nm; fluorescence yield was monitored at 730 nm. Inset: relative height of each peak in the two preparations.

COOPERATION

Cooperation between the Israeli and USA partners and their staff was intensive. This was true for the planning, execution and summarizing levels. Extensive working visits of staff between the two countries took place. Numerous joint publications resulted from this collaboration.

EVALUATION OF ACHIEVEMENTS

The objectives of the project were generally achieved, as evidenced from this report and titles of publications (below) which resulted from the joint work. The results of our research have helped to unravel the controlling signals in the process of photosystem II reaction center turnover *in vivo*. The UV-B studies in particular add a new dimension to the relevance of PS II reaction center turnover for agriculture. Under increased UV-B irradiance, as occurs when stratospheric ozone thins, synergistic light effects may lead to an increase in the rate of reaction center disintegration, causing an energy burden to crop plants.

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