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

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Abstract

The rapidly-metabolized 32kDa protein of the photosynthetic membrane is well known as the target molecule for a wide range of herbicides. It is also recognized as a central component of the photosystem II reaction center. This study deals with rapid, light-dependent turnover of the protein, which is viewed as an important element in understanding both herbicide resistance and oxygenic photosynthetic electron transport.

In this report we identify the in-vivo, light-activated cleavage site of the 32kDa protein. We demonstrate that it is adjacent to the herbicide binding/plastoquinone niche, and borders an alpha-helix-destabilizing region ('PEST' region) common to many rapidly degrading proteins.

Herbicides inhibit both electron transport in photosystem II and 32kDa protein turnover. Utilizing an atrazine-sensitive and -resistant biotype pair, isogenic in the psbA gene except for a single nucleotide, we propose that direct binding of herbicides to the protein (rather than cessation of photosystem II electron transport) is responsible for inhibition of 32kDa protein turnover. Moreover, we find that far red light, specific for photosystem I, can drive herbicide-sensitive, 32kDa protein degradation. We discuss the possibility of degradation involving a semiquinone or free radical step.

Posttranslational modification of proteins can have major effects on activity, conformation or stability of a protein. We show that palmitic acid selectively binds to several chloroplast proteins including, prominently, the 32kDa protein. Bound palmitic acid is associated with the processed form of the 32kDa protein in the granal lamellae. The location of a palmitoylation site specifically in the 'PEST' region near the cleavage site is described.

Original Objectives:

1. To delineate the mechanism by which PSI light is involved in modulating the 32kDa protein. 2. To determine whether the 32kDa protein undergoes fluctuation in response to light intensity, and in this way helps regulate electron flow. 3. To determine if herbicide-inhibition of 32kDa protein degradation is a result of halted electron flow, or, rather, a direct consequence of the binding of the herbicide ligand to the 32kDa protein molecule. 4. To identify the proteolytic breakdown products of the 32kDa protein and whether some of them are stable or functional. 5. To ascertain whether electric polarization is involved in light-intensity-dependent breakdown of the 32kDa protein. 6. To determine the function of post-translational palmitoylation of the 32kDa protein.

Deviations: Objective 2 was abandoned as the topic underwent intensive study by Ohad, Hirschberg and their collaborators. Objective 5 was given low priority by the BARD review board and was abandoned. All the other objectives were addressed.

RESEARCH FINDINGS

CHAPTER 1. INTRODUCTION

OVERVIEW

Chloroplasts encode one of the most studied plant gene products, a unique hydrophobic protein with an apparent molecular weight of 32000 (32K) on SDS-polyacrylamide gels. 32K has a conserved primary structure in all the cyanobacteria, algae and higher plants tested. It is, moreover, a major product of the chloroplast protein synthesizing machinery and one of the most rapidly turned over proteins in plants. 32K is also well known as the monogenic target for the commonly used photosystem II (PSII) herbicides, atrazine and diuron. Finally, it has recently been recognized as an essential component of the oxygenic PSII reaction center. (For current reviews on 32K, see Mattoo et al., 1987; Michel and Deisenhofer 1988; Rochaix and Erickson 1988).

This is a busy protein, with molecules of chlorophyll, pheophytin, non-heme iron and quinone all liganded to it (Nanba and Satoh 1987). PSII herbicides act by displacing the liganded quinone (Q_B) from the protein (Vermaas et al. 1983). In so doing, they block oxygen evolution as well as 32K degradation (Mattoo et al. 1984). We have demonstrated that the primary site of 32K cleavage is adjacent to the Q_B/herbicide binding niche, and contiguous with an alpha-helix destabilizing region (PEST region) common to many rapidly degraded proteins (Greenberg et al. 1987). Interestingly, this region is absent from the analogous, but long-lived, Q_B-binding L subunit of the

non-oxygenic, photosynthetic bacterial reaction center (Michel and Deisenhofer 1988; Mattoo et al. 1988). A tertiary structural model of the Q_B binding niche has been constructed (Trebst 1987) based on genetic (Hirschberg and McIntosh 1983; Rochaix and Erickson 1988) and X-ray crystallographic (Deisenhofer et al. 1985) data. Thus, considerable structural information exists concerning the region of the protein surrounding the light-activated cleavage site. In addition, a start has been made in analyzing activated cleavage process itself (Kyle et al. 1984; Gaba et al. 1987).

32K and its sister protein, D2, are present in stoichiometric amounts. However, 32K is much more rapidly degraded than D2 (Gounaris et al. 1987). We have investigated the 'life cycle' of 32K. The protein is first synthesized as precursor of 33.5 kDa and is associated with the unstacked stromal lamellae (Mattoo et al., 1987). The precursor is then processed at its carboxyl terminus to the mature 32K size (Marder et al., 1984; Eyal et al., 1987). Following processing, 32K translocates to the topologically-distinct granal lamellae (Mattoo et al. 1987). Post-translational modifications of unknown functions, involving palmitoylation (Mattoo et al. 1987) and phosphorylation (Michel et al. 1988), occur. Subsequently, the protein is degraded via a 23.5 kDa intermediate (Greenberg et al. 1987). These studies have revealed a dynamic cycle for 32K, and possibly, for the entire PSII reaction center (Callahan et al. 1987; Mattoo et al. 1989).

Our achievements during the tenure of the current grant include: a) identifying the primary in vivo cleavage site on 32K which is activated by light; b) demonstrating that PSI light, acting alone, can support rapid turnover of 32K; c) establishing that soon after maturation on the stromal lamellae, 32K

translocates to the topologically-distinct granal lamellae; and d) discovering that post-translational palmitoylation of 32K occurs in the region of the primary cleavage site. These studies, among others, are presented in detail in this report.

References

- Callahan, F. E., M. Edelman, A. K. Mattoo, in Progress in Photosynthesis Research, J. Biggens, Ed. (Nijhoff. The Hague, 1987), Vol. 3, p. 799.
- Deisenhofer, J., O. Epp, K. Miki, R. Huber, H. Michel, Nature 318, 618 (1985).
- Eyal, Y., P. Goloubinoff, M. Edelman, Plant Mol. Biol. 8, 337 (1987).
- Gaba, V., J. B. Marder, B. M. Greenberg, A. K. Mattoo, M. Edelman, Plant Physiol. 84, 348 (1987).
- Greenberg, B. M., V. Gaba, A. K. Mattoo, M. Edelman, EMBO J. 6, 2865 (1987).
- Gounaris, K., U. Pick and J. Barber, FEBS Lett. 211, 94 (1987).
- Hirschberg, J. and L. McIntosh, Science 222, 1346 (1983).
- Kyle, D. J., I. Ohad, C. J. Arntzen, Proc. Natl. Acad. Sci. 81, 4070 (1984).
- Marder, J. B., P. Goloubinoff, M. Edelman, J. Biol. Chem. 259, 3900 (1984).

Mattoo, A. K., F. C. Callahan, B. M. Greenberg, P. Goloubinoff, M. Edelman, in
Biotechnology for Crop Protection, P. Hedin et al., Ed. (American Chemical
Society, Washington, D.C., 1988), Chapter 18, p. 248.

Mattoo, A. K., H. Hoffman-Falk, J. B. Marder, M. Edelman, Proc. Natl. Acad.
Sci. U.S.A. 81, 1380 (1984).

Mattoo, A. K., J. B. Marder, M. Edelman, Cell 56, 241 (1989).

Mattoo, A. K. and M. Edelman, Proc. Natl. Acad. Sci. USA, 84, 1497 (1987).

Michel, H., D. F. Hunt, J. Shabanowitz, J. Bennett, J. Biol. Chem. 263, 1123
(1988).

Michel, H., J. Deisenhofer, Biochemistry 27, 1 (1988).

Nanba, O. and K. Satoh, Proc. Natl. Acad. Sci. U.S.A. 84, 109 (1987).

Rochaix, J.-D., J. Erickson, Trends Biochem. Sci. 13, 56 (1988).

Trebst, A., Z. Naturforsch. 42c, 747 (1987).

Vermaas, W. F. J., C. J. Arntzen, L.-Q. Gu, C.-A. Yu, Biochim. Biophys.
Acta 723, 266 (1983).

CHAPTER 2.

CONTROL OF 32kDa THYLAKOID PROTEIN DEGRADATION AS A CONSEQUENCE OF HERBICIDE BINDING TO ITS RECEPTOR

INTRODUCTION

Triazine and phenylurea-type herbicides (such as atrazine and diuron, respectively) inhibit photosynthetic electron transport by competing, at overlapping positions [Trebst, 1979; Pfister et al., 1979], for a quinone binding site [Vermaas et al., 1983] on the rapidly metabolized 32kDa thylakoid protein [Mattoo et al., 1984]. Atrazine resistance has evolved in many weeds [LeBaron and Gressel, 1982] including Solanum nigrum [Gasquez and Barralis, 1979], but these resistant biotypes are still sensitive to diuron. The chloroplast psbA gene coding for the 32kDa protein was recently shown to be isogenic between triazine resistant ('R') and sensitive ('S') S. nigrum biotype pair except for a single nucleotide change in the 'R' phenotype [Goloubinoff et al., 1984]. Electron transport in the S. nigrum 'R' biotype was refractile to the presence of atrazine although effectively blocked by diuron [Gasquez and Barralis, 1979]. The 32kDa herbicide-binding protein is degraded during photosynthetic electron transport and this degradation is inhibited by photosystem II (PS II) herbicides, atrazine and diuron (Mattoo et al., 1984; Mattoo and Edelman, 1985). These data allowed us to present a model linking photosynthetic electron transport to degradation of the 32kDa protein, with PSII herbicides inhibiting both processes (Mattoo et al., 1984; Mattoo and Edelman, 1985).

We have now examined whether herbicide-mediated inhibition of 32kDa protein degradation is a result of halted

electron flow through PSII or, rather, a direct consequence of the binding of the herbicide ligand by the 32kDa protein receptor. First we used 'S' and 'R' biotypes of S. nigrum to confirm our previous data [Mattoo et al., 1984] on the light-dependent catabolism of radiolabeled 32kDa protein and its inhibition in the presence of diuron and atrazine. Next, we took advantage of the different threshold quantum energy requirement for photosystem I (PSI) and PSII and determined if 32kDa protein turnover in Spirodela oligorrhiza was influenced by the part of the far-red spectrum that drives PSI but not PSII. The latter approach yielded data showing that 32kDa protein turnover occurs in the absence of PSII electron transport and that even under these conditions diuron inhibits the 32kDa protein degradation.

RESULTS

The abilities of thylakoid preparations from triazine 'S' and 'R' S. nigrum biotypes to bind radioactive diuron and atrazine are compared in Figure 1. As found for other biotype pairs differing in the psbA gene at codon 264 [Erickson et al., 1985], the 'R' sample had only a mild reduction in diuron binding ability but a strong reduction in atrazine binding compared to the 'S' sample. These data are consistent with published data on other triazine 'R' biotypes (Pfister et al., 1979, 1981; Hirschberg et al., 1984; Erickson et al., 1985) and show that also in the 'R' biotype of S. nigrum the molecular basis for atrazine resistance is a severely lowered binding of this herbicide to the thylakoids.

Catabolism of 32kDa Protein in Triazine Resistant Versus Sensitive S. nigrum Biotypes:

A consistent difference in the kinetics of 32kDa protein degradation in the light was observed between untreated 'S' and 'R' biotypes of S. nigrum and of Amaranthus hybridus. That is, the 32kDa protein in the 'R' biotypes was degraded faster than in the 'S' biotypes. These data suggest that the 32kDa protein in 'R' biotypes is less stable and probably functionally weaker than its counterpart in 'S' biotypes. A slightly different conformation of the 32kDa protein in some 'R' biotypes was revealed by in vitro trypsinization experiments (Mattoo et al., 1982). However, the mechanism for faster degradation of the 32kDa protein in the 'R'

Figure 1. Binding of atrazine and diuron to thylakoids from triazine-resistant ('R') and triazine-sensitive ('S') *Solanum nigrum* biotypes. The binding of herbicides was carried out at 22°C following a 10 min incubation of thylakoids (50 μ g chlorophyll) with [carbonyl- 14 C]-diuron or [ethyl- 14 C]-atrazine in 1 ml of medium as described (Pfister et al., 1979).

biotypes remains to be elucidated.

The effects of diuron and atrazine on degradation of the 32kDa protein in leaf discs of 'R' and 'S' biotypes were then determined. Discs were pulse-labeled with [35 S]methionine, chased in the absence and presence of diuron or atrazine, and the radioactive membrane proteins analyzed by fluorography following SDS-polyacrylamide gel electrophoresis. The distribution of radioactivity as a function of time of chase for untreated samples and those treated with atrazine or diuron revealed that both atrazine and diuron inhibited degradation of the 32kDa protein in the 'S' biotype. However, in samples from the 'R' biotype only

diuron treatment was effective in inhibiting degradation of this protein. Thus, inhibition of 32kDa protein degradation by these two herbicides is coincident with the ability of the biotype to bind the herbicide. In the 'S' biotype both diuron and atrazine bind to the thylakoids and both markedly inhibit 32kDa protein degradation, while in the 'R' biotype only diuron is bound and only diuron prevents 32kDa protein degradation. These findings are in agreement with the hypothesis as illustrated in Figure 2.

Catabolism of 32kDa Protein in Far-red Illumination and the Effect of Diuron:

Spirodela oligorrhiza was pulse-labeled in white light and the radiolabel in membrane proteins was chased for up to 24 h under white light (400-700 nm wavelength) or under far-red illumination (700-730 nm wavelength). PSII luminescence, in the far-red light conditions used, elicited virtually no signal from PSII as compared to that in white light [Marder, 1985]. Under both of these light conditions, the 32kDa protein was degraded at similar rates. In other words, far-red light was as effective as white light in bringing about 32kDa protein degradation. Controls incubated in the dark showed no degradation of the 32kDa protein. Surprisingly, diuron markedly inhibited 32kDa protein degradation in far-red light as well as in white light (Mattoo et al., 1984). This was not anticipated since under far-red illumination, diuron-sensitive PSII reactions did not occur. Thus, 32kDa protein turnover in the light can occur in the absence of photosynthetic electron transport through the diuron-sensitive site, and yet diuron is still effective in inhibiting this process. Taken together, these data give credence to our suggestion that binding of herbicide ligand to 32kDa protein controls turnover of this herbicide receptor protein.

CONCLUSIONS

We have confirmed and extended our previous observation of rapid, light-dependent turnover of the 32kDa thylakoid protein in S. oligorrhiza and the inhibitory effect of diuron and atrazine on this process [Mattoo et al., 1984; Mattoo and Edelman, 1985] to other higher plants, viz. S. nigrum and A. hybridus. We furthermore found that inhibition of 32kDa

Figure 2. Diagrammatic representation of the inhibition of photosynthetic electron flow and degradation of the 32kDa protein by binding of herbicides in triazine sensitive ('S') and resistant ('R') biotypes. In UNTREATED 'S' and 'R' biotypes, undegraded 32kDa protein mediates electron flow. In the process, the protein is degraded in both biotypes. Upon application of diuron (+DIURON), binding (●) to the 32kDa protein ensues in both 'S' and 'R' biotypes. Under these conditions the 32kDa protein is rendered inactive. Concomitantly, electron flow [Gasquez and Barralis, 1979] and degradation of the protein are inhibited in both biotypes. Upon application of atrazine (+ATRAZINE), binding (●) to the 32kDa protein ensues in the 'S', but not 'R', biotype [Pfister et al., 1981]. Concomitantly, electron flow [Gasquez and Barralis, 1979] and degradation of the protein are inhibited only in the 'S' biotype. The basis for the above hypothesis is to be found in this work, in Gasquez and Barralis[1979] and Pfister et al[1981], in Mattoo et al [1984] in which photosynthetic e^- flow was linked to the 32kDa protein degradation, and in Mattoo et al[1981] from which binding of diuron to the 32kDa protein can be inferred.

protein degradation by these two herbicides correlates with the ability of the biotype to bind the specific herbicide.

In addition, our results showing protein degradation in the absence of PSII electron transport, and arrest of 32kDa protein degradation under far-red illumination in the presence of diuron, suggest that diuron- or atrazine-mediated inhibition of 32kDa protein degradation is not likely to be a result of halted PSII electron flow, but rather a direct consequence of the recognition/binding of the PSII herbicides by the protein receptor molecule. Furthermore, we speculate that far-red light may activate a specific protease responsible for 32kDa protein degradation or that the 32kDa protein becomes unstable when it associates with the semiquinone molecules as part of its function in the chloroplast.

REFERENCES

- Erickson JM, Rahire M, Rochaix J-D, Mets L (1985). Herbicide resistance and cross-resistance: Changes at three distinct sites in the herbicide-binding protein. *Science* 228: 204-207.
- Gasquez J, Barralis G (1979). Mise en évidence de la résistance aux triazines chez Solanum nigrum L. et Polygonum lapathifolium L. par observation de la fluorescence de feuilles isolées. *CR Acad Sci Paris Sec D* 288: 1391-1393.
- Goloubinoff P, Edelman M, Hallick RB (1984). Chloroplast-coded atrazine resistance in Solanum nigrum: psbA loci from susceptible and resistant biotypes are isogenic except for a single codon change. *Nuc Acids Res* 24: 9489-9496.
- Hirschberg J, Bleecker A, Kyle DJ, McIntosh L, Arntzen CJ (1984). The molecular basis of triazine-herbicide resistance in higher-plant chloroplasts. *Z Naturforsch* 39c: 412-420.

- Marder JB (1985). Structure and physiology of the rapidly-metabolised 32 kilodalton chloroplast membrane protein. Ph D dissertation, The Weizmann Institute of Science, Rehovot, Israel.
- Mattoo AK, Edelman M (1985). Photoregulation and metabolism of a thylakoidal herbicide-receptor protein. In St. John JB, Jackson PC, Berlin E (eds): "Frontiers of Membrane Research in Agriculture" Totowa, NJ: Roman & Allanheld Publ, pp 23-34.
- Mattoo AK, Hoffman-Falk H, Marder JB, Edelman M (1984). Regulation of protein metabolism: Coupling of photosynthetic electron transport to in vivo degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membranes. Proc Natl Acad Sci USA 81: 1380-1384.
- Mattoo AK, Marder JB, Gressel J, Edelman M. (1982). Presence of the rapidly-labelled 32,000-dalton chloroplast membrane protein in triazine resistant biotypes. FEBS Lett 140: 36-40.
- Mattoo AK, Pick U, Hoffman-Falk H, Edelman M (1981). The rapidly metabolized 32000 dalton polypeptide is the proteinaceous shield regulating photosystem II electron transport and mediating diuron herbicide sensitivity in chloroplasts. Proc Natl Acad Sci USA 78: 1572-1576.
- Pfister K, Radosevich SR, Arntzen CJ (1979). Modification of herbicide binding to photosystem II in two biotypes of Senecio vulgaris L. Plant Physiol 64: 995-999.
- Pfister K, Steinback KE, Gardner G, Arntzen CJ (1981). Photoaffinity labeling of a herbicide receptor protein in chloroplast membranes. Proc Natl Acad Sci USA 78: 981-985.
- Trebst A (1979). Inhibition of photosynthetic electron flow by phenol and diphenylether herbicides in control and trypsin-treated chloroplasts. Z Naturforsch 34C: 986-991.
- Vermass WJF, Arntzen CJ, Gu L-G, Yu C-A (1983). Interactions of herbicides and azoquinones at photosystem II binding site in the thylakoid membrane. Biochim Biophys Acta 723: 266-275.

CHAPTER 3.

Identification of a primary *in vivo* degradation product of the rapidly-turning-over 32 kd protein of photosystem II

Introduction

One of the major products of protein synthesis in chloroplasts is a membrane bound protein of apparent M_r 32,000 (32 kd) (Ellis, 1981; Reisfeld et al, 1982). This protein, also known as the D_1 (Chua and Gillham, 1977) and Q_B -binding protein (Hirschberg et al, 1984), acts on the reducing side of photosystem II (Mattoo, et al, 1981). The 32 kd protein binds triazine and urea herbicides (Gardner, 1981; Steinback et al., 1981; Gressel, 1985; Mattoo et al., 1986). Additionally, the 32 kd protein is proposed to be an integral part of the photosystem II reaction center (Trebst, 1986; Barber and Marder, 1986; Nanba and Satoh, 1987).

The 32 kd protein is synthesized on chloroplast ribosomes as a precursor with an apparent M_r of 33,500 to 34,500 (Grebanier et al, 1979; Ellis, 1981; Reisfeld et al, 1982; Minami and Watanabe, 1985). The precursor protein is then processed at its carboxy terminus to the mature sized protein (Marder et al, 1984). Following processing on the stromal lamellae, the protein is transported to the granal lamellae (Mattoo and Edelman, 1987). One characteristic feature of the 32 kd protein is its rapid degradation (Edelman and Reisfeld, 1978; Silverthorne and Ellis, 1980; Mattoo et al, 1984; Ohad et al, 1985). The rates of synthesis and degradation of the protein are light-intensity (fluence rate) dependent (Mattoo et al, 1984), with turnover occurring in white light or, with reduced efficiency, in far red light, where PSI is predominantly active (Gaba et al, 1987). The mechanism and pathway of light-dependent 32 kd protein turnover are still unclear.

In order to understand the biological roles protein turnover plays, it is important to know how proteins are degraded. Recently, progress has been made in understanding degradation mechanisms by studying factors that target a protein for destruction. It has been found that ubiquitin (Hershko, 1983; Ciechanover et al. 1984), free radicals (Wolff et al, 1986), the amino terminal residue (Bachmair et al, 1986), and certain characteristic amino acid sequences (Rogers et al, 1986) can all play key roles in initiating protein breakdown. We have studied the degradation pathway of the 32 kd protein as a step towards determining the signals responsible for its destruction. Using radiolabeling experiments in conjunction with an antibody against the 32 kd protein, a major *in vivo* breakdown product with an apparent M_r of 23,500 was identified.

Materials and Methods

Culture conditions and radiolabeling.

Spirodela oligorrhiza was grown phototrophically under cool white fluorescent lamps (cf. Hoffman-Falk et al, 1982). Radiolabeling with [^{35}S]-methionine was carried out as previously described (Marder et al, 1984). Labeling times and fluence rates are given in the figure legends. CHI, at 20 $\mu\text{g}/\text{ml}$, was used where indicated. For protein degradation studies, *Spirodela* was radiolabeled with [^{35}S]-methionine and then shifted to a medium containing 1 mM non-radioactive methionine (chase medium) (Mattoo et al, 1984). Fluence rates, labeling times and chase times are given in the figure legends.

Isolation of cell membranes.

Spirodela plants were homogenized, membranes isolated and protein content determined as described by Marder et al (1986). The membranes were washed once in 150 mM NaCl, 2.5 mM tris-glycine (pH 8.5) and once in 2.5 mM tris-glycine (pH 8.5) (Reisfeld et al, 1982).

SDS-PAGE.

Electrophoresis of proteins was carried out according to the procedure of Laemmli (1970) using a 5% stacking gel and a 10-20% gradient gel for separation. Cell membranes were solubilized in sample application buffer (Mattoo et al, 1981) and loaded on the gels on an equal protein basis (10 $\mu\text{g}/\text{lane}$). In all experiments apparent molecular weights (kd) were determined by comparison to [^{14}C]-molecular weight standards (Amersham, CFA-626). Electrophoresis (150 V for 4 hr), fixation (20% methanol, 7% acetic acid), fluorography, exposure on Agfa Curix RP-2 x-ray film, and

quantitation by densitometry were as previously described (Marder et al, 1984). A Beckman Du-8 spectrophotometer with densitometer attachment and an Ambis β -Scanning system were used for quantitation.

Protein blotting and overlay with antiserum.

Proteins from *Spirodela* homogenates were separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose filter (Schleicher and Schuell, BA 83) and treated with antiserum as described (Gershoni and Palade, 1982; Gershoni et al, 1985). An antiserum specific to the the 32 kd protein (Ohad et al, 1985) was hybridized to the filter and detected with [125 I]-Protein A (Amersham).

Partial proteolytic analysis.

Partial proteolysis (Cleveland et al, 1977) was carried out as previously employed (Marder et al, 1984).

Results

Immunological Detection of a 32-kd Protein Breakdown Product.

In an effort to identify polypeptides related to the 32 kd protein, a protein blot of a homogenate from *Spirodela* was probed with a polyclonal antiserum specific to the 32 kd protein. The antiserum cross reacted with the 32 kd protein and with other protein bands with apparent sizes of 52 kd, 29 kd and 23.5 kd upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1, lane 1).

The 32 kd protein has been reported to form aggregates that can be detected by SDS-PAGE (Marder et al, 1984). It is assumed that the immuno-labeled 52 kd band (Figure 1, lane 1) is such an aggregate, and similar to that reported by Sayre et al (1986). The immuno-labeled 29 kd band (Figure 1, lane 1) is probably a conformer of the intact 32 kd protein. This is supported by the following observations: 1. When a co-electrophoresing, [³⁵S]-methionine-labeled 29 kd band was excised and electrophoresed on a second polyacrylamide gel, more than half the material ran with a mobility of 32 kd (data not shown). 2. A 29 kd band is also seen upon *in vitro* translation of SP-6 RNA-polymerase-generated 32 kd mRNA. The *in vivo* as well as *in vitro*-labeled 29 kd polypeptide showed partial proteolytic patterns indistinguishable from that of the 32 kd protein (Eyal, 1986).

The remaining immuno-reactive band, at 23.5 kd, was considered to be a possible breakdown product of the 32 kd protein. If so, its short term synthesis should not be affected significantly by the presence of cycloheximide (CHI) (Edelman et al, 1984). On the other hand, pretreatment with CHI should reduce the level of newly-synthesized cytoplasmic protein and might enable detection of the radiolabeled 23.5 kd polypeptide. Accordingly,

Spirodela plants were radiolabeled with [^{35}S]-methionine in the presence of CHI, and the membrane fraction isolated and analyzed by SDS-PAGE. A polypeptide of 23.5 kd was indeed observed (Figure 1, lane 2), that co-electrophoresed with the polypeptide from the non-CHI treated sample in the immuno-blot (Figure 1, lane 1). This polypeptide appears to be tightly bound to the membrane as it was not detected in the soluble fraction (data not shown). The 23.5 kd band was not easily resolved in control plants radiolabeled in the absence of CHI (Figure 1, lane 3).

Partial proteolysis of the 23.5 kd and 32 kd proteins.

Proteolytic fingerprinting on SDS-polyacrylamide gels (Cleveland et al., 1977) was used to determine if the radiolabeled 23.5 kd polypeptide is related to the 32 kd protein. The major papain digestion products of the 32 kd protein are the polypeptides P20, P12, P10 and P8 (Marder et al., 1984). P20 is derived from the amino-terminal portion of the 32 kd protein while the other products arise, successively, from the carboxy terminus of the protein (Figure 2C). When the 23.5 kd polypeptide was digested with papain, a new band was produced which co-electrophoresed with P20; however, P12 and its digestion products were not seen (Figure 2A).

Digestion of the 32 kd protein with *S. aureus* V8 protease results in bands at 22 kd (Sa22), 21 kd, (Sa21) 10 kd (Sa10) and 8 kd (Sa8) (Reisfeld et al., 1982, Marder et al, 1984). Sa21 is derived from Sa22 and contains P20 entirely within it (Marder et al, 1984) (Figure 2C). Sa10 is a mixture of at least two bands: one is derived from the carboxy terminal region and yields Sa8 upon further proteolysis (Marder et al, 1984), while the remaining band(s) arises from Sa21, which is cut approximately in half at higher protease concentration (J. Marder and M. Edelman, unpublished). When the

23.5 kd polypeptide was incubated with *S. Aureus* V8 protease, Sa22 and Sa21 were formed at low enzyme concentration, while at higher enzyme concentration Sa21 and a 10 kd band were produced (Figure 2B). An additional minor band at 14 kd, which can be seen at higher enzyme concentration, may have arisen by direct proteolysis of the 23.5 kd polypeptide. Alternatively, it may be due to contamination of the initial 23.5 kd band with non-32 kd-related polypeptides.

In summary, the partial proteolysis data support the immunological suggestion of a relationship between the 32 kd protein and the 23.5 kd polypeptide. The presence of P20 and the Sa22-Sa21 doublet in the 23.5 kd polypeptide, on the one hand, and the absence of P8 and Sa8, on the other, show that the 23.5 kd polypeptide corresponds to the amino terminal portion of the 32 kd protein.

Instability of the 23.5 kd polypeptide.

It was previously shown that the 32 kd protein from *Spirodela* is rapidly degraded in the light, but is stable in the dark (Mattoo et al, 1984; Gaba et al, 1987). The radiolabeled 23.5 kd polypeptide was assayed in pulse-chase experiments to determine its metabolic behavior. In Figure 3, it can be seen that the 23.5 kd polypeptide likewise rapidly degrades in the light, and is stable in the dark. Aside from a slower degradation of a 31 kd band (identified as the D2 protein, unpublished), such behavior is unique to the 32 kd and 23.5 kd bands on the autoradiograms. When 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is present, degradation of the 32 kd protein is inhibited (Mattoo et al, 1984), and a reduction in the amount of 23.5 kd polypeptide is observed (data not shown). However, at this stage we do not know whether this effect is due to reduced availability

of the 32 kd substrate to degradation or a direct effect of DCMU on the 23.5 kd polypeptide.

Relative Levels of the 23.5 kd and 32 kd Proteins as a Function of labeling Time.

In the preceding experiments, it was established that the membrane-bound 23.5 kd polypeptide is equivalent to the amino terminal region of the 32 kd protein and is degraded rapidly in the light, but not in the dark. It is now important to determine how the 23.5 kd polypeptide arises. It could be an *in vitro* artifact generated during membrane isolation, the result of premature termination of transcription or translation *in vivo*, or, a true *in vivo* catabolite of the 32 kd protein. The next experiment was designed to distinguish among these three possibilities.

Light-grown *Spirodela* fronds were radiolabeled for various times and the relative amounts of radioactivity in the 23.5 kd and 32 kd bands were quantified (Figure 4). In the early stages of labeling, the ratio between the breakdown product and the 32 kd protein (23.5/32) is approximately 0.1. This ratio increases some three fold with labeling time until it reaches a steady state level after one hour. Such behavior is indicative of an *in vivo* precursor-product relationship. If the 23.5 kd polypeptide was generated *in vitro* during membrane isolation, or resulted from premature termination of synthesis, the 23.5/32 ratio should have remained constant throughout the experiment. Thus, we conclude that the 23.5 kd polypeptide is a true physiological breakdown product of the 32 kd protein.

Discussion

In this paper we describe an *in vivo* breakdown product of the 32 kd protein which has a mobility of 23.5 kd upon SDS-PAGE. The 23.5 kd polypeptide is identified as the amino-terminal region of the parent protein. This segment of the 32 kd protein is hydrophobic (Marder et al, 1984) and probably transverses the thylakoid several times (Trebst, 1986; Barber and Marder, 1986, Rao and Argos, 1986). Thus, as expected, it remains bound to the thylakoid. This is in contrast to the soluble, putative breakdown products that were found in a cell free system from *Chlamydomonas* and ascribed to the 32 kd protein (Wettern and Galling, 1985). The relationship between the 23.5 kd polypeptide and these soluble polypeptides is not known.

Primary cleavage of the 32 kd protein occurs in the hydrophilic loop between helices IV and V.

The position of the cleavage site involved in producing the 23.5 kd polypeptide can be estimated by comparison with the papain fragment, P20 (cf. Figure 2C). The carboxy terminus of P20 is thought to be at, or very near, arg225 (Marder et al, 1984), while its amino terminus has recently been placed about 1 kd from met1 (P. Goloubinoff, personal communication). It is not unreasonable to assume that the 23.5 kd polypeptide arises, *in vivo*, from a single cleavage of its parent protein, as we did not find larger fragments appearing during degradation. If so, cleavage of the 32 kd protein would occur about 2 kd carboxy terminal to arg225; ie., somewhere between amino acid residues 238 and 248, a phylogenetically-conserved (eg. Curtis and Haselkorn [1984]), glutamate rich domain of the 32 kd protein.

Whatever its exact position, the cleavage site is undoubtedly located in the large hydrophilic loop between helices IV and V (see Figure 5) in the

currently favored model of the 32 kd protein (Trebst, 1986; Barber and Marder, 1986; Rao and Argos, 1986; Sayre et al, 1986). This is the region of the protein that is proposed to be involved in quinone and herbicide binding (Wolber et al. 1986) and exposed to the space where the appressed membranes meet (Trebst, 1986). This is also the genetic locale where single amino acid changes confer herbicide resistance (Hirschberg and McIntosh, 1983; Goloubinoff et al, 1984; Erickson et al, 1985; Golden and Haselkorn, 1986; A. Trebst, personal communication).

Is a PEST-like sequence involved in 23.5 kd polypeptide formation?

Additionally, in the loop between helices IV and V, there is a 14 amino acid sequence bounded by arg225 and arg238, which is particularly rich in glutamate, serine and threonine residues (Figure 5). This 14 amino acid stretch of α -helix-destabilizing amino acids has many features in common with 'PEST' regions, recently proposed to be primary determinants for rapid degradation of proteins (Rogers et al, 1986). We note that the carboxy end of this PEST-like sequence is adjacent to the likely primary site of cleavage of the 32 kd protein (Figure 5). A computer search for PEST-like regions failed to yield additional positively scoring regions among more than 50 chloroplast proteins. This included all the identified proteins of the fully-sequenced tobacco chloroplast genome (Shinozaki et al, 1986). Thus, the occurrence of the 'PEST'-like sequence specifically in the rapidly-turning-over 32 kd protein, and, moreover, in the immediate vicinity of its primary site of cleavage, is considered to be significant.

It should be pointed out that the PEST-like region found in the 32 kd protein is lacking a proline. However, an absolute requirement for any individual component within the consensus PEST sequence has not been

established (M. Rechsteiner, personal communication). Proline is known to cause kinks in proteins. Such kinks, along with regions that contain negative charges and hydroxy groups, could be recognized by specific proteases. In the case of the 32 kd protein, a permanent kink in the structure might be replaced by a conformational change following light-mediated damage to the protein. Indeed, the region around the PEST-like sequence in the 32 kd protein has been shown to be more resistant to digestion by trypsin in the dark than in the light (Mattoo et al, 1981). Such a light switch might be essential to limit 32 kd protein turnover in the absence of photodamage.

Neither ATP mediated degradation by ubiquitin conjugation (Hershko, 1983; Ciechanover et al, 1984) nor N-terminal amino acid composition (Bachmair et al., 1986) appear to play a role in the formation of the 23.5 kd cleavage product. ATP is not rate limiting for degradation of the 32 kd protein (Mattoo et al, 1984), while proteins having methionine at their N termini (as does the carboxy-terminally-processed 32 kd protein [Marder et al, 1984]) would be stable according to the N-end rule (Bachmair et al, 1986). It is, however, possible that either of these mechanisms are involved in subsequent degradation of the 23.5 kd polypeptide. On the other hand, the PEST-like sequence, which is likely to be at the carboxy end of the 23.5 kd polypeptide, may still mediate 23.5 kd polypeptide degradation.

Degradation of the 23.5 kd polypeptide is appreciably faster than that of the 32 kd parent protein.

After one hour of labeling, the ratio of the 23.5 kd polypeptide to the 32 kd protein (23.5/32) reaches a steady state level of about 0.28 (cf. Figure 4). Also, net incorporation of radioactivity into the two proteins levels off at this

point. Thus, the concentration of the breakdown product can be described by the reaction:

Where k_1 and k_2 are the apparent rate constants, representing a summation of all the steps involved in degrading the respective proteins. Under steady state conditions, $23.5/32 = k_1/k_2$. Therefore, the rate of degradation of the breakdown product is approximately 4 times that of the parent protein.

As the 23.5 kd polypeptide is itself unstable, it should be considered as an intermediate catabolite in a degradation pathway. Its fate, however, has not been established. A band at 21 kd, which is often observed in chase experiments in *Spirodela* (cf. Figure 3), is being analyzed as to its relationship to the 23.5 kd polypeptide. Also, membrane bound polypeptides in the size range 8- to 14 kd can be detected in thylakoid samples from maize and *Solanum* using the antiserum to the 32 kd protein (data not shown). Some of these may be further breakdown products of the 23.5 kd polypeptide. The fate of the carboxy terminal region of the 32 kd protein remains to be elucidated, as well.

Figure Legends

Fig. 1: Immunodetection and radiolabeling of 32 kd-related proteins in *Spirodela*. **Immunodetection:** Whole cell proteins from *Spirodela* plants were resolved by SDS-PAGE and transferred on to nitrocellulose filter paper. The protein blot was incubated with an antiserum specific to the 32 kd protein (Ohad et al, 1985) and the antigen antibody complex was detected using [125 I]-Protein A (lane 1). Positions of the immunoreacting proteins with apparent sizes of 52, 32, 29 and 23.5 kd are indicated. **In vivo labeling:** *Spirodela* plants were radiolabeled with [35 S]-methionine for 3h in white light at $25 \mu\text{moles m}^{-2} \text{s}^{-1}$ in the presence (lane 2) or absence (lane 3) of CHI. Membrane proteins were isolated, fractionated by SDS-PAGE and detected by autoradiography. Positions of the 32 kd protein and light harvesting chlorophyll protein (LHCP) are marked.

Fig. 2: Partial proteolytic analysis of the 23.5 and 32 kd proteins. The [35 S]-methionine labeled polypeptides at 32 kd and 23.5 kd following SDS-PAGE (cf. Fig. 1, lane 2) were excised and digested with papain (A) or *S. aureus* V8 protease (B) as previously described (Marder et al, 1984). The protease concentrations used (mg/ml) are given above the gel lanes. The arrows below the gel lanes indicate the direction of increasing protease concentration for digestion of the 23.5 kd or 32 kd proteins. Mobilities of the characteristic digestion products are indicated. A map of the 32 kd protein showing positions of the 23.5 kd polypeptide and various protease digestion fragments is presented in panel C. The amino (N) and carboxy termini (C) are marked. Dotted ends indicate uncertainties in the sites of proteolytic digestion.

Fig. 3: Light-dependent degradation of the 23.5 kd polypeptide. *Spirodela* plants were pulse-labeled for 3h with [^{35}S]-methionine in the presence of CHI in white light at $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Control). After radiolabeling, the plants were washed and then placed in chase medium without CHI in the light (L), at a fluence rate of $25 \mu\text{mol m}^{-2} \text{s}^{-1}$, or in the dark (D). Membrane proteins were isolated, separated by SDS-PAGE and detected by autoradiography. Chase times are given in hours (h). The positions of the molecular weight standards, the 32 kd protein and the 23.5 kd polypeptide are indicated.

Fig. 4: Relative accumulation of radioactivity in the 23.5 and 32 kd proteins as a function of time. *Spirodela* plants were radiolabeled with [^{35}S]-methionine in the presence of CHI in white light at $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ for various times. Membrane proteins were separated by SDS-PAGE, detected by fluorography and the relative amounts of the 23.5 kd and 32 kd proteins determined by densitometry. The ratio of radioactivity in the 23.5 kd polypeptide to that in the 32 kd protein (corrected for the reduced number [9] of methionine residues in the 23.5 kd polypeptide vs. that [12] in the 32 kd protein) was then plotted against time. Bars on the graph represent standard errors of the mean for three or more experiments.

Fig. 5: Molecular environment of the 32 kd-protein degradation site. The 32 kd protein is shown spanning the membrane 5 times in analogy to the data of Deisenhofer et al (1985), and as modeled by Trebst (1986) and Barber and Marder (1986). Details of the loop between helices IV and V are presented in the enlargement. The proposed degradation site is shown

as a saw-tooth region. This region is adjacent to the carboxy end of the PEST-like sequence (shown as circled amino-acid residues), while the azidoatrazine binding domain (residues 214 to 225; Wolber et al, [1986]) borders the amino end of the PEST-like sequence. The presently known sites of genetic mutation linked to herbicide tolerance (Hirschberg and McIntosh, 1983; Erickson et al, 1985; A. Trebst, personal communication) are indicated as filled circles. Also shown are histidine residues 215 and 272 which bind the iron and quinone acceptor in the analogous L protein of the *Rhodospseudomonas viridis* reaction center (Deisenhofer et al, 1985). The conserved, higher-plant amino acid sequence for the 32 kd protein (Zurawski et al, 1982) holds for *Spirodela* as well throughout the loop between helices IV and V (B. Greenberg and D. Heller, unpublished).

FIG. 1

FIG 2.

FIG. 3

FIG. 4

FIG. 5

References

- Bachmair,A., Finley,D. and Varshavsky,A. (1986) *Science*, **234**, 179-186.
- Barber,J. and Marder,J.B. (1986) In Russel, G. E. (ed), *Genetic Engineering Reviews*. Intercept, Newcastle-upon-Tyne, pp. 355-404.
- Ceichanover,A., Finley,D. And Varshavsky,A. (1984) *J. Cell. Biochem.*, **24**, 27-53.
- Chua,N.-H. and Gillham,N.W. (1977) *J. Cell Biol.*, **74**, 441-452.
- Cleveland,D.W., Fischer,S.G., Kirschner,M.W. and Laemmli,U.K. (1977) *J. Biol. Chem.*, **252**, 1102-1106.
- Curtis,S.E. and Haselkorn,R. (1984) *Plant Mol. Biol.*, **3**, 249-259.
- Deisenhoffer,J., Epp,O., Miki,K., Huber,R. and Michel,H. (1985) *Nature*, **318**, 618-624.
- Edelman,M., Mattoo,A.K. and Marder,J.B. (1984) In R. J. Ellis (ed.), *Chloroplast Biogenesis*. Cambridge University Press, pp 283-302.
- Edelman,M. and Reisfeld,A. (1978) in Akoyunoglou,G. and Argyroudi-Akoyunoglou,J.H. (eds.), *Chloroplast Development*. Elsevier Biomedical Press, Amsterdam, pp. 641-652.
- Ellis,R.J. (1981) *Ann. Rev. Plant Physiol.*, **32**, 111-137.
- Erickson,J.M., Rahire,M. Rochaix,J.D. and Mets,L. (1985) *Science* **228**, 204-207.

- Eyal,Y. (1986) MSc. Thesis, The Weizmann Institute of Science, Rehovot, Israel.
- Gaba,V., Marder,J.B., Greenberg,B.M., Mattoo,A.K. and Edelman,M. (1987) *Plant Physiol.*, (in press).
- Gardner,G. (1981) *Science*, **211**, 937-940.
- Gershoni,J.M., Davis,F.E. and Palade,G.E. (1985) *Anal. Biochem.*, **144**, 32-40.
- Gershoni,J.M. and Palade,G.E. (1982) *Anal. Biochem.*, **124**, 396-405.
- Golden,S.S. and Haselkorn,R. (1985) *Science*, **229**, 1104-1107.
- Goloubinoff,P., Edelman,M. and Hallick,R.B. (1984) *Nucl. Acids Res.*, **12**, 9489-9496.
- Grebanier,A.E., Steinback,K.E. and Bogorad,L. (1979) *Plant Physiol.*, **63**, 439-439.
- Gressel,J.G. (1985) In S. O. Duke (ed), *Herbicide Physiology*. CRC Press, Boca-Raton, FL, pp. 159-189.
- Hershko,A. (1983) *Cell*, **34**, 11-12.
- Hirschberg,J. and McIntosh,L. (1983) *Science*, **222**, 1346-1349.
- Hirschberg,J., Bleecker,A., Kyle,D.J., McIntosh,L. and Arntzen,C.J. (1984) *Z. Naturforsch.*, **39c**, 412-420.

- Hoffman-Falk,H., Mattoo,A.K., Marder,J.B., Edelman.M. and Ellis,R.J. (1982) *J. Biol Chem.*, **257**, 4583-4587.
- Laemmli,U.K. (1970) *Nature New Biol.*, **227**, 680-685.
- Marder,J.B., Goloubinoff,P. and Edelman,M. (1984) *J. Biol. Chem.*, **259**, 3900-3908.
- Marder,J.B., Mattoo,A.K. and Edelman,M. (1986) *Methods in Enzymol.* **118**, 384-396.
- Mattoo,A.K. and Edelman,M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1497-1501.
- Mattoo,A.K., Hoffman-Falk,H., Marder,J.B. and Edelman,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1380-1384.
- Mattoo,A.K., Marder,J., Gaba,V. and Edelman,M. (1986) In G. Akoyunoglou ed, *Regulation of Chloroplast Differentiation*. Alan R. Liss, Inc. pp607-613
- Mattoo,A.K., Pick,U., Hoffman-Falk,H. and Edelman,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1572-1576.
- Minami,E.-I. and Watanabe,A. (1985) *Plant Cell Physiol.*, **26**, 839-846.
- Nanba,O. and Satoh,K. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 109-112.
- Ohad,I., Kyle,D.J. and Hirschberg,J. (1985) *EMBO J.* **4**, 1655-1659.
- Rao,J.K.M. and Argos,P. (1986) *Biochim. Biophys. Acta*, **869**, 197-214.
- Reisfeld,A., Mattoo,A.K. and Edelman,M. (1982) *Eur. J. Biochem.*, **124**, 125-129.

- Rogers,S., Wells,R. and Rechsteiner,M. (1986) *Science*, **234**, 364-368.
- Sayre,R.T., Anderson,B. and Bogorad,L. (1986) *Cell*, **47**, 601-608.
- Silverthorne,J. and Ellis,R.J. (1980) *Biochim. Biophys. Acta*, **607**, 319-330.
- Shinozaki,K., Ohme,M., Tanaka,M., Wakasugi,T., Hayashida,N., Matsubayashi,T., Zaita,N., Chunwongse,J., Obokata,J., Yamaguchi-Shinozaki,K., Ohto,C., Torazawa,K., Meng,B.Y., Sugita,M., Deno,H., Kamogashira,T., Yamada,K., Kusuda,J., Takaiwa,F., Kato,A., Tohdoh,N., Shimada,H. and Sugiura,M. (1986) *EMBO J.*, **5**, 2043-2049.
- Steinback,K., McIntosh,L., Bogorad,L. and Arntzen,C.J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7463-7467.
- Trebst,A. (1986) *Z. Naturforsch.*, **41c**, 240-245.
- Wettern,M. and Galling,G. (1985) *Planta*, **166**, 474-482.
- Wolber,P.K., Eilmann,M. and Steinback,K.E. (1986) *Arch. Biochem. Biophys.*, **248**, 224-233.
- Wolff,S.P., Garner,A. and Dean,R.T. (1986) *Trends Biochem. Sci.*, **11**, 27-31.
- Zurawski,G., Bohnert,H.J., Whitfield,P.R. and Bottomley,W. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7699-7703.

CHAPTER 4.DEGRADATION OF THE 32kDa HERBICIDE BINDING PROTEIN
IN FAR RED LIGHT*INTRODUCTION*

The rapidly-metabolised 32kDa thylakoid protein has been postulated to function as a regulatory component of PSII (17). It is an integral part of the PSII core complex (18) and, most likely, a reaction center protein (2, 28). The 32kDa protein binds quinones (29) and is the site of action of triazine and diphenyl urea herbicides (9).

In a previous study (16) we showed that the rates of synthesis and degradation of the 32kDa protein are strongly influenced by light intensity (fluence rate). The catabolism of this protein was linked to photosynthetic electron transport, degradation being effectively blocked by PSII inhibitors such as DCMU and atrazine. In line with these findings, we considered it likely that PSII activity would be a specific requirement for catabolism.

This hypothesis was tested by following degradation of the protein in far red (FR)⁴ light. PSI and PSII differ in threshold quantum energy requirements. As a result, PSI can be driven by a narrow band of wavelengths in the far red part of the spectrum in which PSII is inactive. Unexpectedly, it was found that FR light promoted metabolism of the 32kDa protein and that degradation was inhibited by DCMU. These data and their implications are presented here.

MATERIALS AND METHODS

Radiolabeling conditions. Experiments were performed using axenic cultures of *Spirodela oligorrhiza* (Kurtz) Hegelm grown autotrophically on half-strength Hutner's medium (21). All dark manipulations were carried out with the aid of a dim green safelight. Synthesis of the 32kDa protein was determined as follows: radiolabeling was started in darkness by the addition of [^{35}S]methionine (>800 Ci/mmol; Amersham) to a concentration of 0.1 mCi/ml medium. Samples were then incubated for 1h at the required light conditions. Subsequently, plants were washed in the dark with ice-cold distilled water, drained and frozen. Degradation of the 32kDa protein was determined as follows: Plants were radiolabeled for 3h in $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ of cool-white fluorescent light and then distributed to 35 mm Petri dishes each containing 3 ml of chase medium (half-strength Hutner's medium with 1 mM methionine). After an additional hour in chase medium, in white light, the first time point (referred to as zero time chase) was taken. Plants were then incubated for 4, 9 and 18 h at the required light conditions. To stop the reactions, the medium was drained and the plants frozen. Membrane samples were prepared for SDS-PAGE as described by Marder et al (15), and then assayed by fluorography. Loading of all gel lanes within a given experiment was done on an equal protein basis.

Light sources. Cool-white fluorescent tubes were used for white light treatments. FR light was produced by filtering the light from a 500W tungsten halogen floodlamp through a 180 mm thick water filter, a 2 mm thick KG3 (Schott) heat absorbing glass and a layer of Wratten 89B filter (Kodak). The spectral properties of the output are shown in Fig. 4A. Occasionally, an RG 715 filter glass (Schott), which gave a similar spectral

photon distribution, was used in place of the Wratten 89B filter. Long-wavelength far red (L-FR) light was produced by filtering the light from a projector equipped with a tungsten halogen lamp through heat absorbing glass and a sheet of Wratten 88A filter (Kodak) (see Fig. 4A). Light measurements were made with a LiCor Li-188B quantum flux meter/radiometer and spectral photon distributions were measured with a LiCor 1800 spectroradiometer.

Delayed Luminescence. *Spirodela* fronds received a 100 ms flash of either white light ($35 \mu\text{mol m}^{-2} \text{s}^{-1}$) or FR light ($70 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the range 700-730 nm). PSII luminescence was detected by a photomultiplier through a 685 nm interference filter and a shutter timed to open 50 ms after the end of the light flash. The method is described by Malkin (14).

Photoacoustics. This technique directly measures the relative evolution of O_2 from PSII in intact *Spirodela* fronds. Measurements were made according to Bults et al (5).

RESULTS

Metabolism of the 32kDa protein in FR light. Light ≥ 700 nm (FR light) activates PSI but not PSII, while light between 400 and 700 nm (white light) activates both photosystems (23). We compared the effects of FR light on synthesis and degradation of the 32kDa protein with those previously determined by Mattoo et al (16) for white light and darkness. The results in Fig. 1 show that darkness does not support a significant rate of 32kDa protein synthesis, while white light and FR light are both effective in this regard. By comparison, the LHCP is synthesized equally well under all three conditions. This is in agreement with previous data where synthesis

of the LHCP was found to be initially uncoupled from illumination (3).

As seen in Fig. 2, the 32kDa protein is also rapidly degraded in FR light. Mattoo et al. (16) previously showed that in darkness, or in white light in the presence of DCMU, degradation of the 32kDa protein is considerably slowed. The results in Fig. 2 reveal that DCMU likewise suppresses FR-light stimulated degradation of the 32kDa protein. The same was found with atrazine, another PSII inhibitor (data not shown). We note that in FR light (700-730 nm range) a fluence rate of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ was required to achieve a similar rate of 32kDa protein metabolism as that obtained with $12 \mu\text{mol m}^{-2} \text{s}^{-1}$ of white light. Thus, while effective, FR light is less efficient than white light in driving the synthesis and degradation of this protein.

PSII activity is minimal under the FR light conditions used. Metabolism of the 32kDa protein in FR light and the blockage of degradation by PSII-specific inhibitors were not anticipated. Therefore, it was important to establish whether both, or only one, of the photosystems were active *in vivo* under the FR light conditions used. Two separate tests were employed to determine PSII activity in FR light in *Spirodela* fronds. The first was the detection of delayed luminescence after a brief light pulse. Such light emission is known to be almost exclusively from PSII with very little contribution from PSI (14). As seen in Fig. 3A, white light elicits a large response compared to the empty chamber control, whereas the FR light treatment produces a very low response.

Similar results were obtained when oxygen evolution was measured using the photoacoustic technique (Fig. 3B). Modulated 650 nm light produces a large, pulsed oxygen signal which is lost in the presence of the saturating

(unmodulated) light. In contrast, there is very little oxygen evolution in response to the modulated FR light. We calculate that in intact *Spirodela* <3% of the oxygen is evolved in FR light (700-730 nm range) as compared with white light (400-700 nm) on an equal photon basis. When expressed on the basis of equal rates of 32kDa protein destruction under the two light regimes, this value is still <10%. Thus, PSII activity is minimal under the FR light conditions used.

Measurement of PSI-related activity in FR light. PSI activity is difficult to demonstrate directly in *Spirodela* fronds. We employed bleaching by light in the presence of methyl viologen as an indication of PSI activity in these plants. Methyl viologen is reduced by the terminal electron donor of PSI. Bleaching by light is considered to be caused by oxygen radicals which are formed in response to reoxidation of methyl viologen (6, 25). The data in Table 1 show that FR light (≥ 700 nm) induces bleaching of *Spirodela* in the presence of methyl viologen, although not quite as effectively as white light does. However, in L-FR light (>730 nm), where PSI activity is sharply reduced (23), chlorophyll loss is minimal and comparable to the dark control. The source of electrons in *Spirodela* fronds for sustained reduction of methyl viologen in FR light is unknown.

The absence of 32kDa protein degradation in long-wavelength FR light. Degradation of the 32kDa protein was examined using different broad-band far red filters. The spectral characteristics of the light fields which were used are shown in Fig. 4A. FR light promoted degradation of the 32kDa protein, while L-FR light was ineffective (Fig. 4B). Therefore, the 32kDa protein is stable under light conditions where both PSI and PSII are inactive.

DISCUSSION

We have demonstrated that degradation of the 32kDa protein in *Spirodela* fronds can occur as a function of PSI-specific light. We now discuss the means by which FR light causes this process. Several alternatives are considered.

Phytochrome. There is the possibility that a non-photosynthetic photoreceptor such as phytochrome might cause the FR light-induced responses shown here. This possibility is difficult to exclude directly; however, it seems highly unlikely from our data. Continuous high-fluence-rate FR light can elicit phytochrome responses in etiolated tissue, but these have not been found in green tissue such as we have used here (8, 24). There is also little possibility of a threshold response to phytochrome (see ref. 19) in our case. The active form of phytochrome which is found in green plants is stable for many hours in darkness (12). Thus, were it involved, it should be available to cause rapid degradation of the 32kDa protein in darkness. However, such degradation is known to occur at a minimal rate (16).

Plastoquinone pool. A second alternative is that the reduction state of the plastoquinone pool, which modulates certain enzyme activities, could regulate 32kDa protein degradation. For example, a kinase which phosphorylates LHCP (27) is controlled by the redox state of the plastoquinone pool. In the case of the kinase, added FR light reversed the phosphorylation process as it oxidised the plastoquinone pool. However, in our case degradation of the 32kDa protein can be activated under conditions where the plastoquinone pool is either reduced (e.g. in white light) or oxidised (e.g. in FR light). Moreover, in the dark, where degradation is

deactivated, the plastoquinone pool is likewise oxidised. Therefore, it is unlikely that the redox state of the plastoquinone pool is involved in regulating the degradation of the 32kDa protein.

PSI reducing power. A variety of chloroplast enzymes are activated or inactivated by light acting through the photochemical reactions of photosynthesis (4). Activation in some cases may require reducing power from PSI; e.g. malate dehydrogenase and glucose-6-phosphate dehydrogenase, which can be activated either by light or *via* sulphydryl reducing agents (1). Where DCMU inhibited enzyme photoactivation this was ascribed to the blockage of electron flow from PSII to PSI, which is required to maintain PSI reducing power (4). As regards the 32kDa protein, the spectral response for degradation (wavelengths <730 nm) fits in with the known spectral characteristics of photosynthesis. In addition, we have shown that PSI operates in the FR light conditions used here. Thus, 32kDa protein degradation might in fact be a process activated by PSI reducing power. Degradation could involve activation of a specific protease or modification of the 32kDa protein to a degradation sensitive form. The inhibitory effect of DCMU on FR-driven 32kDa protein degradation would then be best understood as protection against protease degradation through direct binding of the inhibitor to the 32kDa protein molecule.

Semiquinone anion. Another possible explanation for FR-light induced destruction of the 32kDa protein is related to its proposed role as a quinone binding protein. This explanation would extend the idea of Kyle et al (13), who proposed that the Q_B semiquinone anion, formed transiently during PSII photoreactions, may inflict some damage on the 32kDa protein environment. The proposal of Kyle et al appears sufficient to explain degradation of the

32kDa protein in white light. DCMU inhibition of white-light driven degradation would be understood since the herbicide is thought to act by displacing Q_B from its binding environment (29). We suggest that FR light might also be expected to induce the formation of the Q_B semiquinone anion. Although photoreduction of plastoquinone is usually thought of as an activity of PSII, cyclic electron flow around PSI also seems to involve redox turnover of the plastoquinone pool (7). Since the plastoquinone reduction step in cyclic electron flow would necessarily involve transient semiquinone formation, there would be the continuous presence of potentially damaging semiquinone molecules even in FR light. Under these conditions DCMU would still protect the 32kDa protein by preventing semiquinone anion binding. Thus, semiquinone-anion inflicted damage might be the activator for 32kDa protein degradation.

Free radicals. Finally, it has been suggested that oxygen free-radicals are generated during electron transport through PSI (10). It is possible that reactive compounds of this nature could cause localized damage to the 32kDa protein resulting in its degradation. Free radicals would also be generated when PSII and PSI are operating together (11, 26), and this as well could be the unifying theme for degradation in FR and white light.

In summary, the mechanism of light-dependent 32kDa protein turnover is still unclear. An *in vitro* 32kDa-protein degradation system (22), amenable to both light and chemical activation, could aid in distinguishing among the various possibilities. However, for a cell-free system to be useful, equivalence with the *in vivo* situation will have to be proved. Although we can conclude that PSI specific light can cause turnover of the 32kDa protein, it would be erroneous to assume that this is the only control operating *in vivo*. There is

probably a major contribution due to linear electron flow (PSII working with PSI), as less white light (approximately 3- to 4-fold) than PSI-specific FR light is required to achieve a given rate of 32kDa protein turnover. Indeed, it is likely that there are several different means to cause the damage that is the fundamental reason for the turnover of this PSII protein. Application of non-photosynthetic light to analyze 32kDa degradation may be helpful in pinpointing the primary mechanism.

TABLE 1. *Bleaching of Spirodela Fronds by Light in the Presence of Methyl Viologen*

Chlorophyll loss due to bleaching by light and methyl viologen (10^{-4} M) was measured after 3 days of incubation. Chlorophyll content was determined by the method of Moran and Porath (20). Results are expressed as mean percentages of the control $\pm$ se. Fluence rates were: white light ($12 \mu\text{mol m}^{-2} \text{s}^{-1}$), FR light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the range 700-730 nm), and L-FR light ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$).

	% of Control ^a	n ^b
White light	12 $\pm$ 1	9
FR light	24 $\pm$ 3	7
L-FR light	98 $\pm$ 8	3
Darkness	108 $\pm$ 10	10

^aControl fronds incubated without methyl viologen were analyzed for chlorophyll content under each light condition.

^bNumber of repeats.

LEGENDS TO FIGURES

Figure 1. Synthesis of the 32kDa protein in FR light. Radiolabeling with [^{35}S]methionine was carried out for 1h. Proteins were analysed by SDS-PAGE and fluorography. Fluence rates in white light were $12 \mu\text{mol m}^{-2} \text{s}^{-1}$ and in FR light $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ (in the range 700-730 nm). Spectral properties of the light sources are described in Materials and Methods. W, white light; FR, far red light; D, darkness. The positions of the 32kDa protein and LHCP are indicated.

Figure. 2. Degradation of the 32kDa protein in FR light. Pulse labeling of *Spirodela* with [^{35}S]methionine was carried out for 3 h in white light. The radioactivity was then chased from fronds incubated in far red light (FR) or darkness (D) for the times (in hours) indicated above the gel lanes. The zero (0) time control is described in Materials and Methods. Proteins were analysed by SDS-PAGE and fluorography. The Fluence rates during the chase were as described in the legend to Fig. 1. DCMU, at a final concentration of $10\mu\text{M}$, was added to some plants during the chase period as indicated.

Figure 3. Absence of PSII activity in FR light. A. Delayed luminescence. The relative intensity of the light emitted after a white light (W) or far red light (FR) pulse was measured as described in Materials and Methods. The control is the luminescence of the empty sample compartment in FR light. B. Photoacoustic technique. Pulses of O_2 evolved from *Spirodela* fronds were measured in response to a modulated (chopped) light, either 650 nm or FR ($\geq 700 \text{ nm}$). Fluence rates were $35 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 650 nm and $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ (in the range 700-730 nm) (FR). Measurements were made according to Bults et al (5).

Figure 4. *A.* Relative spectral photon distribution of the far-red light sources used. FR light was generated using Wratten filter 89B as described in Materials and Methods. 50% of the photons from the 700-800 nm waveband of the 89B source are in the range 700-730 nm. L-FR light was generated using Wratten filter 88A as described in Materials and Methods.

B. Spectral dependence of 32kDa protein degradation in far red light. The effects of the far-red light sources, described in *A*, on 32kDa protein degradation were examined. The fluence rates in both cases were $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the range 700-800 nm. Other experimental conditions are as described in the legend to Fig. 2. A fluorograph following SDS-PAGE is shown.

FIG. 1

FIG 2.

FIG 3.

FIG 4.

REFERENCES

1. Anderson LE, SC Nehrlich, ML Champigny 1978 Light modulation of enzyme activity: Activation of the light effect mediators by reduction and modulation of the enzyme activity by thio-disulfide exchange. *Plant Physiol* 61: 601-605
2. Barber J, JB Marder 1986 Photosynthesis and the application of molecular genetics. *In* GE Russel ed, *Genetic Engineering Reviews*, Intercept, Newcastle-upon-Tyne, pp 355-404
3. Bennett J 1981 Biosynthesis of the light-harvesting chlorophyll a/b-protein. Polypeptide turnover in darkness. *Eur J Biochem* 118: 61-70
4. Buchanan BB 1980 Role of light in the regulation of chloroplast enzymes. *Ann Rev Plant Physiol* 31: 41-74
5. Bults G, BA Horwitz, S Malkin, D Cahen 1982 Photoacoustic measurement of photosynthetic activities in whole leaves: photochemistry and gas exchange. *Biochim Biophys Acta* 679: 452-465
6. Calderbank A, P Slade 1976 Diquat and Paraquat. *In* PC Kearney, DD Kaufman, eds, *Herbicides Ed 2, Vol 2*. Marcel Dekker, NY and Basel, pp 501-540
7. Crofts AR, CJ Wraight 1983 The electrochemical domain of photosynthesis. *Biochim Biophys Acta* 726: 149-185

8. Gaba V, M Black 1983 The control of cell growth by light. *In* W Shropshire Jr, H Mohr, eds, Photomorphogenesis, Encyclopedia of Plant Physiology, New series, Vol 16. Springer, Berlin, pp 358-400
9. Gressel JG 1985 Herbicide tolerance and alteration of site activity. *In* SO Duke ed, Herbicide Physiology, CRC Press, Boca-Raton, FL, pp 159-189
10. Halliwell B 1981 Toxic effects of oxygen on plant tissue. *In* Chloroplast Metabolism, Clarendon Press, Oxford, pp 179-205
11. Heath RL, L Packer 1968 Photoperoxidation in isolated chloroplasts II. Role of electron transfer. *Arch Biochem Biophys* 125: 850-857
12. Jabben M, MG Holmes 1983 Phytochrome in light-grown plants *In* W Shropshire Jr, H Mohr eds, Photomorphogenesis, Encyclopedia of Plant Physiology, New Series, Vol 16. Springer, Berlin, pp 704-722
13. Kyle DJ, I Ohad, CJ Arntzen 1984 Membrane protein damage and repair: Selective loss of quinone-protein function in chloroplast membranes. *Proc Natl Acad Sci USA* 81: 4070-4074
14. Malkin S 1977 Delayed luminescence. *In* J Barber ed, Primary Processes in Photosynthesis. Elsevier/North-Holland Press, Amsterdam, pp 351-431
15. Marder JB, AK Mattoo, M Edelman 1986 Identification and characterization of the psbA gene product: The 32-kDa chloroplast membrane protein. *Methods in Enzymol* 118: 384-396

16. Mattoo AK, H Hoffman-Falk, JB Marder, M Edelman 1984 Regulation of protein metabolism: coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membrane. Proc Natl Acad Sci USA 81: 1380-1384
17. Mattoo AK, U Pick, H Hoffman-Falk, M Edelman 1981 The rapidly metabolized 32000 dalton polypeptide is the proteinaceous shield regulating photosystem II electron transport and mediating diuron sensitivity in chloroplasts. Proc Natl Acad Sci USA 78: 1572-1576
18. Millner PA, JB Marder, K Gounaris, J Barber 1986 Localization and identification of phosphoproteins within the photosystem two core of higher plant thylakoid membranes. Biochim Biophys Acta (in press)
19. Mohr H 1972 Enzyme repression, mediated by phytochrome through a threshold mechanism. *in* Lectures on Photomorphogenesis. Springer, Berlin, pp 69-76
20. Moran R, D Porath 1980 Chlorophyll determination in intact tissues using N-N'-dimethyl formamide. Plant Physiol 65: 478-479
21. Posner HB 1967 Aquatic vascular plants. *In* FA Witt, NK Wessels, eds, Methods in Developmental Biology. Crowell, NY, pp 301-317
22. Reisman S, I Ohad 1986 Light-dependent degradation of the thylakoid 32 kDa Q_B protein in isolated chloroplast membranes of *Chlamydomonas reinhardtii*. Biochim Biophys Acta 849: 51-61

23. Ried A 1972 Improved action spectra of light reaction I and II. *In* G Forti, M Avron, A Melandri eds, Proc 2nd Internatl Cong Photosynth Res. Dr W Junk, The Hague, pp 763-772
24. Smith H 1975 The photomorphogenic response systems and their photoreceptors. *In* Phytochrome and Photomorphogenesis. McGraw-Hill, London, pp 22-53
25. Summers LA 1980 Plant physiology and mode of herbicidal action. *In* The Bipyridinium Herbicides. Academic Press, London, pp 300-343
26. Takahama U, M Nishimura 1975 Formation of singlet molecular oxygen in illuminated chloroplasts: Effects on photoinactivation and lipid peroxidation. *Plant Cell Physiol* 16: 737-748
27. Telfer A, JF Allen, J Barber, J Bennett 1983 Thylakoid protein phosphorylation during state 1-state 2 transitions in osmotically shocked pea chloroplasts. *Biochim Biophys Acta* 722: 176-181
28. Trebst A 1986 The topology of the plastoquinone and herbicide binding peptides of photosystem II in the thylakoid membrane. *Z Naturforsch* 41C: 240-245
29. Vermaas WFJ, CJ Arntzen, L-Q Gu, C-A Yu 1983 Interaction of herbicides and azoquinones at photosystem II binding in thylakoid membranes. *Biochim Biophys Acta* 723: 266-275

Intra-membrane translocation and posttranslational palmitoylation of the chloroplast 32kDa herbicide binding protein

INTRODUCTION

The internal membranes (thylakoids) of the chloroplast constitute the sites where transformation of solar energy into chemical energy is catalyzed by multisubunit complexes composed of pigments, lipids and proteins. The structural organization of the thylakoids includes granal (stacked) lamellae, which contain mainly photosystem II components, and interconnecting stromal (unstacked) lamellae, which preferentially house the photosystem I components and the ATPase complex(1-4). In apparent apposition to this spatial separation(1,2), there seems to be a functional cooperation between the two photosystems in regulating light energy distribution and photosynthetic electron transport(4,5). Indeed, a phosphorylation-dependent shuttling of mobile, light-harvesting chlorophyll protein complex between the photosystems has recently been proposed to mediate this cooperation(4). However, little is known about the molecular events that target newly synthesized components of the photosynthetic complexes to specific loci within the thylakoids. The 32,000 dalton (32kDa) herbicide binding protein (also referred to as peak D(6), D1 protein(7) and Q_B protein(8)) is a well-studied integral component of photosystem II(9-11). This rapidly-metabolized(6,12) thylakoid protein is a major, light-driven(13) translation product of the chloroplast genome. It contains overlapping binding sites for several classes of herbicides, including the s-triazines, ureas and phenylureas(14). The 32kDa protein is first synthesized as a 33,500-34,500 dalton (33.5kDa) precursor polypeptide(6,15,16). Both the precursor and mature protein are membrane-associated(15,16). The 32kDa protein acts at the electron accepting side of photosystem II(17), possibly as a reaction center component(11,18,19).

Circumstantial evidence has recently accumulated suggesting dual membrane locations for the 32kDa protein. In particular, the discovery of two pools

of 32kDa protein molecules in Chlamydomonas(20), the reported absence of the 32kDa protein from the granal (but not stromal) lamellae in a photosystem II mutant of maize(21), and the demonstration that azidoatrazine binds to a 32kDa protein only in the granal lamellae(22) raised the possibility that some spatial relocation event was a part of the life cycle of the protein. In this study we demonstrate that the 33.5kDa precursor protein is exclusively associated with unstacked stromal lamellae. Processing to the mature 32kDa form occurs in this membrane fraction as well. Following processing a translocation of 32kDa protein to the stacked granal lamellae takes place.

We examined the possibility that posttranslational modification of the 32kDa protein is involved in its membranal deployment. Reversible, covalent modifications such as glycosylation, methylation, phosphorylation and acylation can have major effects on the activity, conformation or stability of a protein(23). In plants, glycosylation of seed storage proteins(24) and phosphorylation of chloroplast membrane proteins(4,25) are well known. Reports on protein acylation(26,27) however, have been mainly confined to viral glycoproteins(28) and a number of bacterial lipoproteins(29,30), viral transforming proteins(31-33) and animal membrane proteins(34-37). Acylation of plant proteins has not been reported. Yet, interactions between lipids and protein complexes are known to occur in the thylakoid. In fact, polar lipids and their fatty acid constituents play an important role in the architecture(38,39) and, probably, function(40) of photosynthetic membranes.

In this study we show that palmitic acid selectively binds to several chloroplast proteins including, prominently, the 32kDa protein. Bound palmitic acid is exclusively associated with the processed 32kDa form, and is readily observed in the stacked granal fraction. At least one palmitoylation

site is present in a membrane protected region of the protein.

MATERIALS AND METHODS

Axenic cultures of S. oligorrhiza were used in all experiments. Conditions of growth, in vivo radiolabeling, isolation of thylakoids and their fractionation into granal and stromal lamellae, and NaDodSO₄/polyacrylamide gel electrophoresis are described in detail in the legends to figures.

RESULTS

Synthesis, processing and translocation of the 32kDa protein. Ellis(41) and others(42,44) have indicated that synthesis of the 32kDa protein, a core component of photosystem II, occurs on membrane-bound chloroplast ribosomes. As a class, these ribosomes were shown to be enriched in the unstacked stromal lamellae(44). However, photosystem II is known to be concentrated in the stacked granal lamellae(4). This conflict can be cogitatively resolved by postulating spatially distinct sites for 32kDa protein synthesis and function. Indeed, in recent studies using fractionated lamellae such a model has been assumed(21,22). We have directly tested this model by following the distribution of newly synthesized 33.5kDa precursor and 32kDa processed protein forms in fractionated lamellae. The biological system employed was Spirodela oligorrhiza, an aquatic higher plant adept at rapidly incorporating nutrient precursors from the underside of its leaf-like frond into macromolecules.

Spirodela plants were radiolabeled with ³⁵S-methionine for 3 minutes, and the radioactivity was chased by further incubating the plants in excess non-radioactive methionine. Figure 1 shows the steady-state (stain) and newly-synthesized (fluorogram) polypeptide profiles of stromal and granal

lamellae following 0, 6 and 120 minutes of chase. The stained gel revealed the distinct polypeptide compositions characteristic of granal and stromal lamellae(4,21): enrichment of photosystem II polypeptides (p47,p43,LHCP) in the granal lamellae, and photosystem I (cpI) and ATPase proteins (α and β) in the stromal lamellae. Electron micrographs showed the stromal lamellar fraction to consist of single-membrane vesicles and the granal lamellar fraction of double membrane sheets. The degree of purity in each fraction approached 95 percent (W.P. Wergin, Beltsville Agricultural Research Center, data not shown).

The fluorogram in Fig. 1 shows that after 3 minutes of label (i.e., 0 time chase) virtually all of the radioactivity in the membrane was in the stromal lamellar fraction, with the 33.5kDa precursor polypeptide being the most prominent band. Processing of the precursor polypeptide to the 32kDa protein also occurred in the stromal lamellae (6 minute chase). However, by 120 minutes little radioactivity remained in this fraction. On the other hand, accumulation of radioactivity in the granal lamellae commenced only after 6 minutes of chase with the processed 32kDa protein being the only prominent band. By 120 minutes of chase virtually all of the radioactivity associated with the 3-minute-pulsed plants was concentrated in the 32kDa protein band of the granal lamellae. These data demonstrate that membrane attachment and processing of the 33.5kDa precursor polypeptide occur in the stromal lamellae. However, once processed, the 32kDa protein is translocated to the topologically-distinct granal lamellae, where it remains at least for 2 hours.

Posttranslational palmitoylation of chloroplast proteins. Post-translational acylation of viral and mammalian proteins has variously been implicated in mediating polypeptide transport and processing, anchoring of

proteins in the membrane, and assembly of protein complexes(26,27). We took note of the high lipid content of the chloroplast and sought to determine if an acylation signal was involved in regulating some aspect of 32 kDa protein organization within the membrane. To this end, Spirodela plants were pulse-labeled in the light for 3 minutes with ^3H palmitic acid, the major saturated-fatty-acid species in the plastid. Membrane and soluble fractions were then prepared and analyzed by SDS-PAGE. Figure 2 shows the distribution of the ^3H -labeled and steady-state (stained) protein bands. Two prominent radioactive bands were seen in the membrane fraction.

One coelectrophoresed with ^{35}S -methionine-labeled 32kDa protein and the other with the high-abundance, light-harvesting chlorophyll a/b protein (LHCP). Some of the radioactivity associated with the membrane fraction migrated near the gel front. Two prominent radioactive bands were also seen in the soluble fraction. One coelectrophoresed with the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxygenase, and the other showed an M_r of 10,000 (10kDa), similar to that of acyl carrier protein (ACP)(45). The identities of three of the major bands were verified in further experiments: trypsinization of isolated ^3H -labeled membranes revealed the characteristic pattern(46) for the 32kDa protein (see Fig. 6); antibodies raised against ribulose-1,5-bisphosphate carboxylase/oxygenase(47) specifically precipitated a ^3H -labeled polypeptide coelectrophoresing with authentic LS (data not shown), while antibodies raised against soybean acyl carrier protein(45) specifically precipitated a ^3H -labeled polypeptide having an electrophoretic mobility of 10kDa and coelectrophoresing with authentic acyl-ACP (Mattoo et al, unpublished).

Figure 3 compares in vivo labeling of proteins with ^3H -palmitic acid in the light and in darkness. The uptake of ^3H -palmitate by Spirodela fronds

during 1 minute of incubation in the light was similar to that in the dark (1.6×10^8 versus 1.2×10^8 cpm/mg chlorophyll, respectively). However, the intensities of the four prominent ^3H -labeled protein bands were considerably greater in the light incubated samples. In analyzing these data we took note of the fact that light also stimulates synthesis of several of these proteins(12,13). However, at least for the 32kDa protein, any increase in the amount of protein due to synthesis in the light, during the 1 minute labeling period, would be unlikely to account for stimulation of protein acylation. This is because palmitoylation occurs at least 3 to 6 minutes after 33.5kDa protein synthesis (cf.Fig. 5). Thus, the association of palmitic acid with these proteins is a bona fide, light stimulated event.

The rapidity with which Spirodela proteins become labeled with ^3H -palmitate in the light (1 to 3 minutes) minimized the risk of metabolism of the labeled moiety. Nonetheless, we carried out experiments to directly identify the radiolabel in the protein bands following polyacrylamide gel electrophoresis. Figure 4 compares the migration of methyl palmitate, authentic ^3H -palmitic acid and the ^3H radioactivity extracted from the putative LHCP gel band hydrolyzed in methanolic HCl. The chromatographic migration of the major peak of radioactivity from the gel band matched those of the palmitate standards. In other experiments, using high-pressure liquid chromatographic analysis of the ^3H -radioactivity extracted from the 32kDa protein gel band, the labeled moiety was identified as 16:0. Moreover, analysis of ^3H -palmitate distribution among thylakoid lipids by gas chromatography following even 60 minutes of label showed 99.8 percent of the radioactivity remaining as 16:0 while 0.2% was recovered at 16:1. No label was detected as myristic acid (Callahan, Norman, Mattoo and St.John, unpublished). These data indicate that the ^3H moiety in the rapidly-

labeled protein bands remains as palmitic acid. Finally, some plants were incubated for 1 to 3 minutes with either ^3H -palmitic acid, ^3H -myristic acid or ^3H -glycerol, and membrane proteins fractionated by SDS-PAGE. Although a few proteins were labeled with ^3H -myristic acid, the intensity of label was minimal compared to that seen with ^3H -palmitic acid (Callahan, Norman, St. John and Mattoo, unpublished). No radioactive protein bands were observed in samples incubated with ^3H -glycerol. These results suggest that palmitic acid is a major fatty acid ligand for acylation of proteins in the chloroplast.

The type of acyl linkage to the chloroplast proteins was investigated by incubating SDS-polyacrylamide gels in 1M NH_2OH at pH 6.5. As seen in Fig. 2, the ^3H -palmitate-labeled 10kDa protein band was sensitive to acidic hydroxylamine, indicating a thiol ester linkage. The remaining ^3H -labeled bands were resistant to this treatment, suggesting a stronger oxygen ether linkage(35).

Palmitoylation of the 32kDa protein in relation to its translocation.

Palmitoylation of the 32kDa herbicide binding protein was studied in some detail. Spirodela plants were labeled with either ^{35}S -methionine for 6 minutes or ^3H -palmitate for 3 minutes, and granal (G) and stromal (S) lamellae were isolated from whole thylakoids. Figure 5 shows the distinctive, stained protein pattern obtained following SDS-PAGE for each lamellar type, as well as two fluorographic exposures of the gel. In agreement with the results shown in Fig. 1, the distribution of the methionine label indicates that synthesis and processing of the 33.5kDa precursor polypeptide occur exclusively at the stromal lamellae. Palmitoylation, on the other hand, is strictly limited to the mature 32kDa

protein band and is associated with the granal lamellae (cf 4 weeks exposure).

The membrane-associated 32kDa herbicide binding protein was previously shown to be partially susceptible to digestion by trypsin(15,17), with a large fragment of the polypeptide protected by the thylakoids(48). Figure 6 shows the SDS-PAGE patterns obtained following partial trypsin digestion of granal lamellae isolated from Spirodela plants labeled with ^{35}S -methionine or ^3H -palmitic acid. The two characteristic membrane-associated fragments, T22 and T20(46), were observed in both cases. This suggests that an acylation site on the 32kDa protein is in the membrane-protected region of the protein.

DISCUSSION

Spatio-temporal dynamics of the 32kDa herbicide binding protein in the chloroplast membranes. Several novel features concerning the 32kDa herbicide binding protein have been demonstrated in this report. These include the subcellular site of processing, membrane translocation and posttranslational acylation. Interrelationships among these and other dynamic membrane events associated with the metabolism of the 32kDa protein are outlined in Fig. 7. A pivotal occurrence is intra-thylakoidal translocation. This is an early event in the life span of the protein and results in a physical separation between the site of precursor processing and the likely major site of 32kDa protein function. Interestingly, a 30kDa polypeptide, most probably the D2 protein, another photosystem II core protein(11,19,49), likewise undergoes translocation from stromal to granal lamellae(50). Perhaps spatial relocation within the thylakoid is a general feature of chloroplast-coded photosystem II proteins.

Superimposed on these movements is the palmitoylation of the 32kDa protein (Fig. 7). It is tempting to suggest that palmitoylation of the processed protein may promote its proper functional integration in photosystem II complex (51) once translocation has occurred. Experiments using inhibitors of acylation and of translocation are now in progress to test this idea. We have also recently determined that palmitoylation of the 32kDa protein is a relatively short-lived event, with deacylation occurring rapidly in the granal lamellae (Callahan, Mattoo and Edelman, unpublished). Nonetheless, present data do not permit assigning this acylation to a precise temporal or even spatial stage in the life cycle of the protein. For example, while the data in Fig. 5 clearly fail to reveal palmitoylated protein in the stromal lamellae, we cannot rule out its ephemeral existence at this location. In such a case, protein modification might serve primarily as a tag leading to rapid translocation of the 32kDa protein to the grana.

The other three chloroplast proteins identified here as undergoing palmitoylation in intact plants are, like the 32kDa protein, matured forms of higher-molecular-weight precursor polypeptides(45,52,53). This fact tends to preclude a role for protein palmitoylation in the transport of the nuclear-encoded LHCP or ACP precursor polypeptides to the chloroplast or in their subsequent processing. Likewise, an involvement in the processing of the plastid-encoded LS protein seems unlikely. The physiological role of palmitoylation in all these cases awaits further study.

Figure legends

Fig. 1. Intra-thylakoid translocation of the 32kDa protein following its synthesis and processing on stromal lamellae. Spirodela oligorrhiza (Kurtz) Hegelm plants were routinely grown phototrophically (25°C, 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, cool white fluorescent lamps) for 10-15 days as axenic cultures on half-strength Hutner's mineral medium(54) containing 0.5% sucrose. For pulse labeling, these cultures were transferred for 2-3 days to mineral medium lacking sucrose. The plants were labeled for 3 min with 660 μCi ^{35}S -methionine in 1 ml of mineral medium, and then washed immediately in non-radioactive mineral medium containing 1mM methionine (chase medium). Samples were removed after 0, 6 and 120 min of incubation in chase medium and each mixed with 2-3 gm fresh weight of unlabeled plants. Whole thylakoids were isolated and fractionated into granal and stromal lamellae as described by Leto et al(21). Polypeptides associated with these membranes were analyzed on 10-20% gradient SDS-polyacrylamide gels as previously described(17). The gels were stained with Comassie blue R (0.2% in 50% methanol-7% acetic acid), destained in 20% methanol and 7% acetic acid(46), fluorographed using Enhance (New England Nuclear), dried and exposed on curix RP2 x-ray films (Agfa). The positions of the photosystem I reaction center polypeptide (cpI), α and β subunits of proton ATPase (α, β), photosystem II polypeptides (p47, p43 and light harvesting chlorophyll protein complex (LHCP)) are indicated on the stained gel, while the positions of the radiolabeled 33.5kDa precursor polypeptide (33.5) and the processed 32kDa protein (32) are indicated on the fluorogram.

Fig. 2. In vivo labeling of Spirodela proteins with ^3H -palmitic acid. Plants were grown in the light as described in the legend to Fig. 1. The plants were radiolabeled in the light ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 25°C) for 3 min with 700-1000 μCi of 9,10- ^3H (N)-palmitic acid (15.2 Ci/mmol; New England Nuclear) in 1 ml of mineral medium. Prior to use, radioactive palmitic acid solution was dried under a stream of nitrogen gas and then dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the labeling medium was kept under 1%. After labeling, the plants were washed twice with 5-10 ml of mineral medium and immediately frozen on dry ice until used. Cell homogenization and fractionation into soluble and membrane proteins was carried out as previously described(16). Samples for gel electrophoresis were mixed with application buffer(17), fractionated by SDS-PAGE and stained with Coomassie blue as described in the legend to Fig. 1. The gels were then destained in 25% isopropanol and 10% acetic acid(31), fluorographed using En 3 Hance, dried and exposed on curix RP2 x-ray films. The stability of the ^3H -acyl linkage was tested as follows. Duplicate gels were run. After fixation, one replicate was washed in water to remove acetic acid and incubated for 24h at 25°C with 1M $\text{NH}_2\text{OH}\cdot\text{HCl}$ (pH 6.6)(31). The second gel was incubated without $\text{NH}_2\text{OH}\cdot\text{HCl}$. Both gels were then dried and fluorographed. The positions of large (LS) and small (SS) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase, the 10kDa acyl carrier protein (10K), the light harvesting chlorophyll a/b apoprotein (LHCP) and the 32kDa herbicide binding protein (32K) are indicated.

Fig. 3. Light-stimulated ^3H -palmitic acid labeling of chloroplast proteins. Phototropically grown *Spirodela* plants were labeled for 1 min in the dark (D) or in the light at $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (L) with ^3H -palmitic acid, washed with 5 ml of mineral medium and immediately frozen on dry ice. Soluble and membrane fractions were isolated, and analyzed by SDS-PAGE as described in the legend to Fig. 1. The positions of prominent ^3H -palmitic acid labeled proteins are indicated.

Fig. 4. Identification of unmodified ^3H -palmitic acid as the radioactive ligand labeling putative LHCP. The ^3H -palmitic acid-labeled gel band coelectrophoresing with authentic LHCP was excised from a dried SDS-polyacrylamide gel, hydrated and homogenized to extract radiolabeled proteins as previously described(31). Hydrolysis was carried out at 90°C in 2 M HCl in 83% methanol for 5-6 hr. Lipophilic components were then solubilized by extraction with petroleum ether. The ether-soluble material was dried in a stream of N_2 , redissolved in petroleum ether and analyzed by thin layer chromatography on a silica gel G plate in parallel with authentic methyl palmitate and ^3H -palmitic acid. The position of methyl palmitate was identified by charring the plate at 100°C after spraying with 50% H_2SO_4 . The distribution of radioactive palmitic acid was determined by dividing the plate into twenty 1 cm strips, scraping the silica gel and counting in a scintillation spectrometer. A typical run is shown for the ^3H -palmitic-acid label released from the putative LHCP band.

Fig. 5. ^3H -palmitic-acid-labeled 32kDa protein is associated with granal lamellae. Spirodela plants were radiolabeled either with ^3H -palmitic acid for 3 min or with ^{35}S -methionine for 6 min in the light as described in the legends to Figs. 1 and 2. Isolation and fractionation of granal (G) and stromal (S) lamellae were as described by Leto et al(21). SDS-PAGE, staining and fluorography were as described in the legend to Fig. 1. The positions of the 33.5kDa precursor polypeptide (33.5) and 32kDa herbicide binding protein (32) in the ^3H -palmitate- and ^{35}S -methionine-labeled gel lanes are indicated. Short (5 days) and longer (4 weeks) exposures of the gel are shown. The positions of marker polypeptides (cf legend to Fig. 1) are indicated.

Fig. 6. ^3H -palmitic-acid-labeling of the 32kDa protein occurs in the trypsin-resistant amino-terminal region. Spirodela plants were labeled in the light either with ^3H -palmitic acid for 3 min, as described in the legend to Fig. 2, or for 60 min with 660 μCi of ^{35}S -methionine in 1 ml of mineral medium. Granal lamellae were isolated(21) and incubated with or without trypsin (1 μg enzyme per μg chlorophyll equivalent of membrane) for 30 min as previously described(46). The two, characteristic membrane-associated tryptic fragments (T22 and T20) of the 32kDa protein(46) are evident in both the ^{35}S - and ^3H -labeled samples.

Fig. 7. Membrane associated events in the life of the 32kDa herbicide binding protein. All time values represent estimated half-times for the protein in *Spirodela* cultured at $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. Light-dependent synthesis(12,13) of nascent 33.5kDa precursor polypeptide takes place on 70S thylakoid-bound ribosomes(41-43), most likely in the unstacked stromal lamellae(44). Following completion of translation, the 33.5kDa polypeptide is associated exclusively with the stromal lamellae (Figs. 1 and 5). At this location, carboxy-terminal processing(48) occurs with a $t_{0.5}$ of 3-6 min(16) (Figs. 1 and 5). Following processing, the 32kDa protein is translocated within the thylakoids, to the stacked, granal lamellae with a $t_{0.5}$ of 6-12 min (Fig. 1). At this new location, where photosystem II is primarily concentrated(4,21), the 32kDa protein is thought to be functionally active and gains the ability to bind herbicides(22). The $t_{0.5}$ of the 32kDa protein, which is thought to normally remain in the granal lamellae, is 6-12 h(55). Thus, the protein spends the majority of its life span in the granal lamellae. Light-intensity dependent degradation of the 32kDa protein(12) occurs in the granal lamellae with production of a 23.5kDa membrane-bound catabolic intermediate(56). At some stage(s) between 32kDa protein translocation and formation of the catabolic intermediate, light-induced (Fig. 3) palmitoylation of the amino terminal region of the protein occurs (Figs. 5 and 6). This tight binding (Fig. 2) of fatty acid to the 32kDa protein is a short-lived event ($t_{0.5}$ about 3-6 min)(50). Hypotheses concerning the functional role(s) of palmitoylation in the life cycle of the 32kDa protein are discussed in the text.

FIG 1.

FIG 2.

FIG 3

FIG 4.

FIG 5.

FIG 6.

FIG 7.

REFERENCES

1. Miller, K. R. & Staehelin, L. A. (1976) *J. Cell Biol.* 68, 30-47.
2. Jennings, R. C., Garlaschi, F. M., Gerda, P.D. & Forti, G. (1979) *Biochim. Biophys. Acta* 456, 207-219.
3. Andersson, B. & Anderson, J. M. (1980) *Biochim. Biophys. Acta* 593: 427-440.
4. Staehelin, L. A. & Arntzen, C. J. (1983) *J. Cell Biol.* 97, 1327-1337.
5. Anderson, J. M. & Melis, A. (1983) *Proc. Natn. Acad. Sci. U.S.A.* 80, 745-749
6. Ellis, R. J. (1981). *Ann Rev. Plant Physiol.* 32, 111-137.
7. Chua, N.-H, & Gillham, N. (1977) *J. Cell Biol.* 74, 441-452.
8. Hirschberg, J., Bleecker, A., Kyle, D.J., McIntosh, L., & Arntzen, C.J. (1984) *Z. Naturforsch.* 39C, 412-420.
9. Mattoo, A.K. & Edelman, M. (1985) in *Frontiers of Membrane Research in Agriculture* (eds St. John, J.B., Berlin, E. & Jackson, P.C.) 23-34 (Rowan & Allanheld, Totowa).
10. Kyle, D. J. (1985) *Photochem. Photobiol.* 41, 107-116.
11. Trebst, A. (1986) *Z. Naturforsch.* 41C, 240-245.
12. Mattoo, A. K., Hoffman-Falk, H., Marder, J.B. & Edelman, M. (1984) *Proc. Natn. Acad. Sci. U.S.A.* 81, 1380-1384.
13. Fromm, H., Devic, M., Fluhr, R. & Edelman, M. (1985) *Embo J.* 4, 291-295.
14. Oettmeier, W. & Trebst, A. (1983) in *The Oxygen Evolving System of Photosynthesis* (ed. Inoue, Y.) 411-420 (Academic Press, Tokyo).
15. Grebanier, A.E., Coen, D.M., Rich A. & Bogorad, L. (1978) *J. Cell Biol.* 78, 734-746.
16. Reisfeld, A., Mattoo, A. K. & Edelman, M. (1982) *Eur. J. Biochem.* 124, 125-129.

17. Mattoo, A.K., Pick, U., Hoffman-Falk, H. & Edelman, M. (1981) *Proc. Natn. Acad. Sci. U.S.A.* 78, 1572-1576.
18. Deisenhofer, J., Epp, O., Miki, K., Huber, R. & Michel, H. (1985) *Nature* 318, 618-624.
19. Barber, J. & Marder, J.B. (1986) in *Biotechnology and Genetic Engineering Reviews* (ed. Russel, G.E.) 355-404 (Intercept Ltd, Newcastle upon Tyne, England).
20. Wettren, M. & Ohad, I. (1984) *Israel J. Bot.* 33, 253-263.
21. Leto, K. J., Bell, E. & McIntosh, L. (1985) *Embo J.* 4, 1645-1653.
22. Wettren, M. (1986) *Plant Science Lett.* 43, 173-177.
23. Wold, F. (1981) *Ann. Rev. Biochem.* 50, 783-814.
24. Higgins, T.J.V. (1984) *Ann. Rev. Plant Physiol.* 35, 191-221.
25. Bennett, J. (1977) *Nature* 269, 344-346.
26. Schmidt, M.F.G. (1982) *Trends. Biochem. Sci.* 7, 322-324.
27. Magee, A.I. & Schlesinger, M.J. (1982) *Biochim. Biophys. Acta* 694, 279-289.
28. Schmidt, M.F.G. (1982) *Virology* 116, 327-338.
29. Braun, V. (1975) *Biochim. Biophys. Acta* 415, 335-377.
30. Tokunaga, M., Tokunaga, H. & Wu, H.C. (1982) *Proc. Natn. Acad. Sci. U.S.A.* 79, 2255-2259.
31. Sefton, B.M., Trowbridge, I.S., Cooper, J.A. & Scolnik, E.M. (1982) *Cell* 31, 465-474.
32. Garber, E.A., Krueger, J.G., Hanafusa, H. & Goldberg, A.R. (1983) *Nature* 302, 161-163.
33. Grand, R.J.A., Roberts, C. & Gallimore, P.H. (1985) *FEBS Lett* 181, 229-235.
34. Slomiany, A., Witas, H., Aono, M. & Slomiany, B. (1983) *J. Biol. Chem.* 258, 8535-8538.
35. Bolanowski, M.A., Earles, B.J. & Lennarz, W.J. (1984) *J. Biol. Chem.* 259,

36. O'Brien, P.J. & Zatz, M. (1984) *J. Biol. Chem.* 259, 5054-5057.
37. Soric, J. & Gordon, J.A. (1985) *Science* 230, 563-566.
38. Quinn, P.J. & Williams, W.P. (1983) *Biochim. Biophys. Acta* 737, 223-266.
39. Remy R., Tremolieres, Duval, J.C., Ambard-Bretteville, F. & Dubacq, J.P. (1982) *FEBS Lett* 137, 271-275.
40. Farineau, N., Guillot-Solomon, T., Tuquet, C. & Farineau, J. (1984) *Photochem. Photobiol.* 40, 387-390.
41. Ellis, R. J. (1977) *Biochim. Biophys. Acta. Review* 463, 185-215.
42. Minami, E-I and Watanabe, A. (1984) *Arch. Biochem. Biophys.* 235, 562-570.
43. Herrin, D. & Michaelis, A. (1985) *FEBS Lett.* 184, 90-95.
44. Yamamoto, T., Burke, J., Autz, G. and Jagendorf, A.T. (1981) *Plant Physiol.* 67, 940-949.
45. Ohlrogge, J. B. & Kuo, T. M. (1985) *J. Biol. Chem.* 260, 8032-8037.
46. Marder, J.B., Mattoo, A.K. & Edelman, M. (1986) *Methods Enzymol.* 118, 384-396.
47. Rabinowitz, H., Reisfeld, A., Sagher, D. & Edelman, M. (1975) *Plant Physiol.* 56, 345-350.
48. Marder, J. B., Goloubinoff, P. & Edelman, M. (1984) *J. Biol. Chem.* 259, 3900-3908.
49. Rochaix, J.-D., Dron, M., Rahire, M. & Malnoe, P. (1984) *Plant Molec. Biol.* 3, 363-370.
50. Callahan, F.E., Edelman, M. & Mattoo, A.K. (1986) in *Proc. VII Intl. Cong. Photosynthesis*, Rhode Island, USA, Abstract #202/264.
51. Liveanu, V., Yocum, C. F. & Nelson, N. (1986) *J. Biol. Chem.* 261, 5296-5300.
52. Tobin, E.M. (1981) *Plant Physiol.* 67, 1078-1083.

53. Langridge, P. (1981) FEBS Lett. 123, 85-89.
54. Posner, H.B. (1967) in Methods in Developmental Biology (eds Witt, F. A. & Wessels, N.K.) 301-317 (Crowell, New York).
55. Edelman, M., Mattoo, A.K. & Marder, J.B. (1984) in Chloroplast Biogenesis (ed. Ellis, R.J.) 282-302 (Cambridge Univ. Press, London).
56. Greenberg, B., Mattoo, A.K., Gaba, V. & Edelman, M. (1986) Plant Physiol. Suppl. 80, 47.

Description of Cooperation

Cooperation between the Israeli and American investigators and their research staff was intensive. It manifested itself at the planning, execution and summarizing levels of the research. This was brought about by 1) open communication channels (BITNET and FAX facilities) directly in each laboratory, and 2) yearly working visits of each side (senior investigators, as well as postdocs and students) to the other's laboratory. The cooperation culminated in frequent joint publications during the grant period (see List of Publications).

Evaluation of Research Achievements

One objective was to delineate the relationship between PSI light and 32k turnover in the PSII reaction center. This was done in a controlled study analyzing the phenomenon, and in indepth discussion of the possible mechanisms involved (Gaba et al., 1987). A second objective was to explore the relationship among photosynthetic electron transport, 32k protein turnover and herbicide inhibition of both processes. This was done in an early study involving atrazine resistant and sensitive biotypes isogenic for the psbA gene except for a single point mutation. A mechanism, favoring direct binding of the herbicide to the 32k molecule was proposed (Mattoo et al., 1986). This work is continuing along more quantitative lines. A third objective was to identify one or more 32k degradation product. The discovery and localization of the primary light-inducing in vivo cleavage site in the molecule was a major achievement (Greenberg et al., 1987) with implications for continuing research on the mechanism of degradation. A forth objective was to study the phenomenon of posttranslational palmitoylation of the 32k protein. This led to a detailed

picture of the life history of the protein in the photosynthetic membrane, and the integration of the palmitoylation signal therein. The location of a palmitoylation site in the vicinity of the primary cleavage site of the protein was described (Mattoo et al. 1987).

The research described in this report helps to provide a deeper understanding of the control of the light reactions in photosynthesis. 32k protein turnover in nature is a massive event with a half-time of about 30 min in sunlight. Being the very heart of the PSII reaction center, the average crop plant would not make it through the day without a regulatory mechanism for maintaining a 32k-PSII reaction center balance. It appears a worthwhile endeavor to study and understand this regulation. Our biological-biochemical studies, along with the biophysical and structural studies of others, have been instrumental in poising 32k research at the molecular genetic level, where specific mechanisms concerning herbicide resistance and efficiency of electron transport can be tested. The long term benefit to agriculture from the basic research studies carried out under this grant is viewed by us in this context.

List of Publications

1. Marder, J. B., Mattoo, A. K. and Edelman, M. Identification and characterization of the psbA gene product: the 32-kDa chloroplast membrane protein. *Methods in Enzymology* 118:384-396, 1986.
2. Mattoo, A. K., Marder, J. B., Gaba, V. and Edelman, M. Control of the 32kDa thylakoid protein degradation as a consequence of herbicide binding to its receptor. In: *Regulation of Chloroplast Differentiation* (G. Akoyunoglou and H. Senger, ed.). "Plant Biology" Series, Alan R. Liss, Inc., Publ., New York, pp. 607-613, 1986.
3. Callahan, F. E., Edelman, M. and Mattoo, A. K. Posttranslational acylation and intra-thylakoid translocation of specific chloroplast proteins. In: *Progress in Photosynthesis Research* (J. Biggins, ed.), Martinus Nijhoff Publ., Dordrecht, The Netherlands. Vol. 3, pp. 799-802, 1987.
4. Mattoo, A. K. and Edelman, M. Intramembrane translocation and postranslational palmitoylation of the chloroplast 32kDa herbicide-binding protein. *Proc. Natl. Acad. Sci. USA* 84:1497-1501, 1987.
5. Gaba, V., Marder, J. B., Greenberg, B. M., Mattoo, A. K. and Edelman, M. Degradation of the 32kDa herbicide binding protein in far red light. *Plant Physiol.* 84:348-352, 1987.

6. Greenberg, B. M., Gaba, V., Mattoo, A. K. and Edelman, M. Identification of a primary in vivo degradation product of the rapidly-turning-over 32 kD protein of photosystem II. EMBO J. 6:2865-2869, 1987.
7. Norman, H. A., St. John, J. B., Callahan, F. E., Mattoo, A. K. and Wergin, J. P. Lipid molecular species composition of granal and stromal lamellae. In: The Metabolism, Structure, and Function of Plant Lipids. (Stumpf, P. K., Mudd, J. B. and Nes, W. D., eds.). Plenum, New York, pp. 193-195, 1987.
8. Mattoo, A. K., Norman, H. A., Callahan, F. E., St. John, J. B. and Edelman, M. Plant protein acylation: identification of the modified proteins and analysis of the bound fatty acid ligand. In: Post-Translational Modification of Proteins by Lipids, A Laboratory Manual, (Brodbeck, U. and Bordier, C., eds.), Les Diablerets, Switzerland, pp. 82-87, 1988.
9. Mattoo, A. K., Callahan, F. E., Greenberg, B. M., Goloubinoff, P. and Edelman, M. Molecular dynamics of the 32,000-dalton photosystem II herbicide binding protein. In: Biotechnology for Crop Protection (Hedin, P., Menn, J. and Hollingsworth, R., eds.), ACS Symposium Series, ACS Books, Washington, D.C., pp. 248-257, 1988.
10. Mattoo, A. K., Marder, J. B. and Edelman, M. Dynamics of the photosystem II reaction center. Cell 56:241-246, 1989.

11. Mattoo, A. K., Callahan, F. E., Mehta, R. A. and Ohlrogge, J. B. Rapid in vivo acylation of acyl carrier protein with exogenous fatty acids in Spirodela oligorrhiza. Plant Physiol. 89:707-711, 1989.
12. Mattoo, A. K., Callahan, F. E., Sopory, S. K. and Edelman, M. Trafficking and distribution of the photosynthetic reaction center proteins in the chloroplast membranes. In: Photosynthesis: Molecular Biology and Bioenergetics (Singhal, et al., eds.), Narosa Pub. House, New Delhi, pp. 189-193, 1989.
13. Greenberg, B. M., Gaba, V., Mattoo, A. K. and Edelman, M. Degradation of the 32kDa photosystem II reaction center protein in UV, visible and far red light occurs through a common 23.5 kDa intermediate. Z. Naturforsch. 44c:77-79, 1989.