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Regulation of protein metabolism: Coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membranes

(*Spirodela oligorrhiza*/light intensity/protein turnover/diuron/atrazine)

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ABSTRACT **In *Spirodela oligorrhiza*, mature chloroplasts copiously synthesize and degrade a 32-kilodalton membrane protein. The rates of synthesis and degradation are controlled by light intensity, the protein being unstable in the light and stable in the dark. Light-driven synthesis, but not degradation, is dependent on ATP. Degradation is blocked by herbicides inhibiting photosystem II electron transport, such as diuron and atrazine. Thus, both anabolism and catabolism of the 32-kilodalton protein are photoregulated, with degradation coupled to electron transport rather than phosphorylation.**

Measurements of protein turnover in plants have, in general, lagged behind those in animals and microorganisms, possibly due to persistent problems of storage and metabolic pools in many plant systems (1). Thus, although a number of studies have focused on light-regulated synthesis of mRNAs and proteins coded by, or expressed in, the chloroplasts (2–5), the possibility of light-regulated degradation of chloroplast proteins has received limited attention. Except for recent studies demonstrating enhanced turnover of the light-harvesting chlorophyll (LHC) a/b apoprotein in darkness (6, 7) and degradation of ribulose bisphosphate (RuP_2) carboxylase/oxygenase during dark-induced senescence or dedifferentiation (8), little is known.

A rapidly synthesized 32-kilodalton (kDa) membrane protein (termed the 32-kDa protein) associated with photosystem II has received increasing attention as a ubiquitous (9) and major (10–12) product of the chloroplasts. It is a target site for several triazine herbicides (13, 14) and a possible binding protein for HCO_3^- or CO_2 (ref. 15). Its chloroplast gene sequence (16), mRNA properties (5, 17, 18), and its carboxyl-terminal processing (19) from a 33.5-kDa precursor form (12) have been described. The 32-kDa polypeptide is hydrophobic and tightly anchored in the photosynthetic membranes (12); however, it also contains hydrophilic sequences that extend into the stroma (13, 20). Proteolytic digestion of the surface-exposed sequences *in vitro*, or depletion of the protein *in vivo*, selectively affects electron transport at the reducing side of photosystem II (13). We have shown this polypeptide to have many properties in common with the allosteric, regulatory protein shield (13) proposed (21–23) to act between the primary and secondary quencher of photosystem II and now consider the 32-kDa protein to form at least a part of that entity.

Although the 32-kDa protein is rapidly synthesized under normal phototrophic conditions, it does not accumulate (24, 25), being degraded some 50 times more rapidly than the large subunit of RuP_2 carboxylase/oxygenase or the LHC protein (26). We have undertaken a study of the factors governing the rate of metabolism of the 32-kDa protein in the aquatic angiosperm *Spirodela oligorrhiza*. Our findings point to the metabolism of the 32-kDa protein as being tightly photoregulated with degradation coupled to photosynthetic electron transport rather than phosphorylation.

MATERIALS AND METHODS

Axenic cultures of *S. oligorrhiza* were used in all experiments (27). Conditions of growth, *in vitro* radiolabeling, membrane isolation, and $NaDodSO_4$/polyacrylamide gel electrophoresis are described in detail in the legends to figures. ATP content was measured by the luceferin-luciferase method (28), whereas oxygen evolution was determined photoacoustically (29).

RESULTS

Requirements for Synthesis of the 32-kDa Protein. *S. oligorrhiza* is a small (1–3 mm) water plant well suited for investigating *in vivo* synthesis and molecular control of chloroplast proteins (25). A mature, green frond placed in complete darkness retains its chlorophyll and photosynthetic membrane structure for up to 3 months, whereas newly developing fronds emerge essentially devoid of chlorophyll and contain etioplasts with few granal stacks (30). Upon exposure to light, and after a lag period of 20–24 hr, the white fronds begin rapid greening, reaching the steady-state light-grown condition within a further 2–3 days (31). Fig. 1 shows the $NaDodSO_4$/polyacrylamide gel distribution of newly synthesized proteins extracted from *Spirodela* fronds after growth in complete darkness for 30 days. During 1 hr of labeling in the dark with [^{35}S]methionine, synthesis of a 33.5-kDa polypeptide was evident (lower arrow, Fig. 1), but neither white nor green fronds synthesized much new 32-kDa protein. This was also the case for white fronds reintroduced to light after the 30-day dark period and then pulse-labeled for 1 hr. However, dark-adapted green fronds, under these same conditions, resumed their normal, light-grown rate of 32-kDa protein synthesis. We conclude from these experiments that light is necessary but not sufficient for inducing rapid synthesis of the 32-kDa protein in *Spirodela*.

In addition to a light requirement for 32-kDa protein synthesis, there appears to be a further requirement for matured chloroplasts. This was revealed by an experiment in which fronds, cultured in darkness for 100 days, were re-exposed to light for increasing periods of time. Membrane polypeptides synthesized during a short-term [^{35}S]methionine pulse were then analyzed on polyacrylamide gels (Fig. 2). Greening of white fronds due to chlorophyll synthesis began by 23

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Abbreviations: kDa, kilodalton(s); LHC, light-harvesting chlorophyll; RuP_2, ribulose bisphosphate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

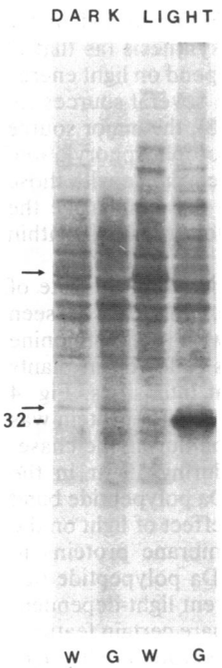

FIG. 1. Synthesis of the 32-kDa protein in dark-adapted green fronds of *Spirodela*. *S. oligorrhiza* (Kurtz) Hegelm was cultured axenically for 8 days under steady-state light (2000 lux, cool-white, 25°C) in half-strength Hutner's mineral medium (27) supplemented with 1% sucrose and then transferred to the dark (25°C). After 29 days of growth in complete darkness, colonies of fronds were transferred aseptically, using a green safety light, into Petri dishes containing fresh, sucrose-free mineral medium and left in darkness for an additional day. Cultures were then radiolabeled for 1 hr either in the dark (designated "DARK") or under 2500-lux illumination (designated "LIGHT") with 70 μCi (2.59 MBq) of [^{35}S]methionine (>1000 Ci/mmol; Amersham) per ml. Labeled plants, in the dark or in the light, were washed with ice-cold mineral medium and dissected in the light at 0°C into individual white (W) and green (G) fronds. Cell homogenates were prepared and proteins were separated by electrophoresis on a 10–20% NaDodSO$_4$/polyacrylamide gradient gel (12). Fifty-thousand cpm of hot trichloroacetic acid-precipitable radioactivity was applied to each lane. The gel was prepared for fluorography and exposed on CURIX RP 2 x-ray film (Agfa) at −70°C. The position of the 32-kDa protein is indicated. The upper arrow indicates the position of the large subunit of RuP_2 carboxylase/oxygenase, whose synthesis in white fronds preceded that in green fronds. The lower arrow shows the position of the 33.5-kDa precursor to the 32-kDa protein, which is maintained at a basal rate of synthesis even in the dark.

hr (arrow, Fig. 2). By 30 hr of light exposure, white fronds initiated significant synthesis of the LHC protein, the major structural component of the photosynthetic membranes. However, synthesis of the 32-kDa protein did not assume massive proportions until after 72 hr of exposure to light, by which time chloroplasts had matured (30). On the other hand, in the dark-adapted green fronds, 32-kDa protein synthesis was induced within 1 hr of exposure to light and preceded synthesis of the LHC protein.

The *in vivo* results with *Spirodela* shown in Fig. 2 complement previous *in organello* studies with greening pea-shoot plastids in which light-driven synthesis of the 32-kDa protein was shown to increase as greening progressed, until this protein became the major translation product (32). Indeed, late acquisition of 32-kDa protein-synthesizing capacity characterized not only greening of etiolated tissues but also normal ontogeny in *Spirodela* (33, 34) and spinach (35). The late appearance of the 32-kDa protein seemed not to be plastid-ribosome dependent, because the large subunit of RuP_2 carboxylase/oxygenase could be synthesized at considerable rates in juvenile (33) and etiolated (32) tissues. Rather, the physical presence of developed membranes, on which the precursor to the 32-kDa protein can be synthesized (12, 36) and into which the mature protein can integrate (19), would seem to be a prerequisite for copious light-driven synthesis.

Metabolism Is Light-Intensity Dependent. The rate of synthesis of the 32-kDa protein in *Spirodela* is related to the intensity of light reaching the plant. This can be seen from Fig. 3, in which incorporation of radioactivity into samples containing equal amounts of membrane proteins was com-

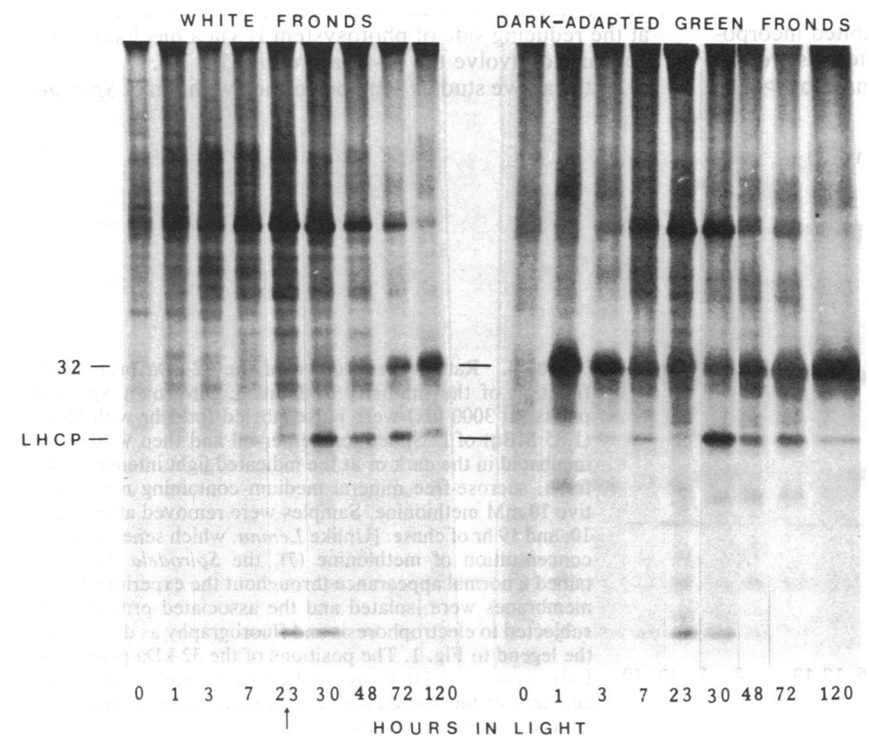

FIG. 2. Comparison of radiolabeled membrane proteins of white and dark-adapted green fronds of *Spirodela* after their exposure to light. *Spirodela* plants were grown in the light and transferred to the dark as described in the legend to Fig. 1. After incubation in complete darkness for 96 days, colonies were transferred to fresh mineral medium without sucrose and left in the dark for 4 additional days before illumination under 2000 lux. Samples were pulse-labeled with 300 μCi (11.1 MBq) of [^{35}S]methionine per ml for 1 hr at the end of the darkness period (lanes 0) or after exposure to light for the times indicated. After washing and dissection, cell membranes were isolated (12) and membrane-associated proteins were subjected to electrophoresis as described in the legend to Fig. 1. Greening of white fronds took place by 23 hr (arrow). The positions of the 32-kDa protein and LHC protein (LHCP) are indicated. Each sample applied on the gels contained 25,000 cpm of hot trichloracetic acid-precipitable radioactivity.

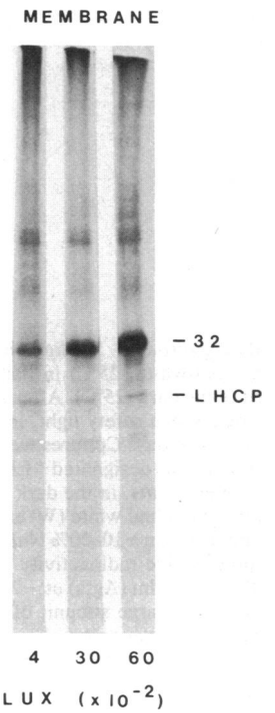

FIG. 3. Rate of synthesis of the 32-kDa protein as a function of the intensity of light. Light-grown *Spirodela* plants (at 2000 lux) were pulse-labeled for 30 min with 350 μCi (12.95 MBq) of [^{35}S]methionine per ml in fresh, sucrose-free mineral medium. Light intensities during labeling were maintained at 400, 3000, or 6000 lux. After labeling, mature fronds were collected and membrane fractions were isolated. Samples with equal amounts of protein were subjected to electrophoresis and fluorography as described in the legend to Fig. 1. The positions of the 32-kDa protein and LHC protein (LHCP) are indicated.

pared for plants incubated at several light intensities. An increased rate of synthesis was found for the 32-kDa protein but not for the LHC protein.

Addition of 5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a phosphorylation uncoupler, to the growth medium of *Spirodela* cultures inhibited incorporation of [^{35}S]methionine into the 32-kDa protein as well as the large subunit of RuP_2 carboxylase/oxygenase by >95% versus control fronds during a 2-hr labeling period in the light. Thus, the rate of 32-kDa protein synthesis (as that of other chloroplast proteins) appears to depend on light energy acting through ATP synthesis. Although several sources for chloroplastic ATP do exist *in vivo* (37, 38), the major source in mature, light-grown tissue is from photophosphorylation. Thus, our findings in *Spirodela* are in accordance with those made for isolated pea chloroplasts (36) and emphasize the role of light as an energy source for protein synthesis within the photosynthetic organelle.

Light intensity has a profound effect also on the rate of degradation of the 32-kDa polypeptide. This can be seen when light-grown *Spirodela* are pulsed with [^{35}S]methionine and loss of label from protein bands is followed in plants incubated in the dark or at various light intensities. Fig. 4 shows that the rate of breakdown of the 32-kDa protein was dependent on the intensity of illumination during the chase. No appreciable breakdown occurred during 19 hr in the dark, while at 6000 lux, label in the 32-kDa polypeptide band was no longer detectable after 5 hr. The effect of light on the degradation of other pulse-labeled membrane proteins in *Spirodela* was minor, except for a 30-kDa polypeptide (arrow, Fig. 4) that apparently also underwent light-dependent breakdown. This polypeptide seems to share certain features with the 32-kDa protein (unpublished observations); its identity is not known.

Catabolism Depends on Photosynthetic Electron Transport. *Spirodela* plants were pulse-labeled with [^{35}S]methionine and chased with nonradioactive methionine for 13 hr in the presence of 5 μM FCCP (Fig. 5). In contrast to synthesis, no change was found in the rate of 32-kDa protein breakdown versus control plants. Thus, degradation of the 32-kDa protein is not dependent on energy from photophosphorylation. Instead, it appears to rely on light energy acting through photosynthetic electron transport. This is illustrated by results with plants chased in the presence of the herbicide diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. A marked retardation in breakdown of the 32-kDa protein is evident versus control plants (Fig. 5). Analogous findings (not shown) were obtained with pulse-labeled *Spirodela* plants chased in the presence of 10 μM atrazine [2-chloro-4-(2-propylamino)-6-ethylamino-*s*-triazine]. In studies with isolated thylakoids, both of these herbicides inhibit photosynthetic electron flow at the reducing side of photosystem II via a mechanism that appears to involve the 32-kDa protein (13, 14).

As the above studies were performed with intact *Spirodela*

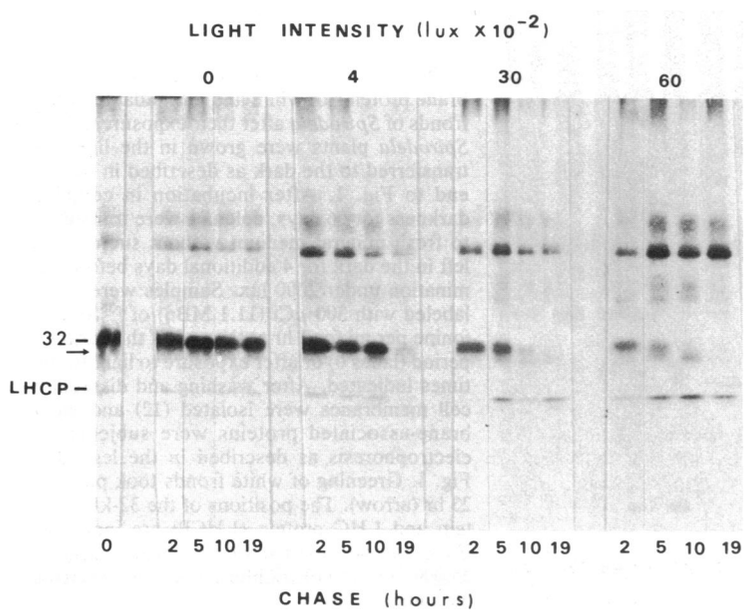

FIG. 4. Rate of breakdown of the 32-kDa protein as a function of the intensity of light. Light-grown *Spirodela* plants (at 3000 lux) were pulse-labeled for 3 hr with 50 μCi (1.85 MBq) of [^{35}S]methionine per ml and then washed and incubated in the dark or at the indicated light intensities with fresh, sucrose-free mineral medium containing nonradioactive 10 mM methionine. Samples were removed after 0, 2, 5, 10, and 19 hr of chase. [Unlike *Lemna*, which senesces at this concentration of methionine (7), the *Spirodela* fronds retained a normal appearance throughout the experiment.] Cell membranes were isolated and the associated proteins were subjected to electrophoresis and fluorography as described in the legend to Fig. 1. The positions of the 32-kDa protein and LHC protein (LHCP) are indicated. Samples with equal amounts of hot trichloracetic acid-precipitable radioactivity (50,000 cpm) were loaded on the gels.

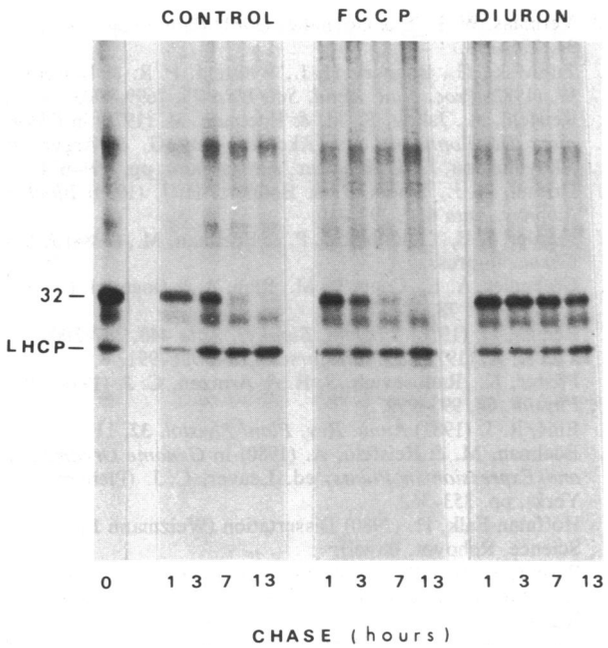

FIG. 5. Degradation of the 32-kDa protein in the presence of FCCP and diuron. Light-grown *Spirodela* plants were transferred to fresh, sucrose-free mineral medium, incubated overnight in the light at 3000 lux, and pulse-labeled at this light intensity for 4 hr with 50 μCi (1.85 MBq) of [^{35}S]methionine per ml. The plants were then washed and resuspended in sucrose-free mineral medium containing nonradioactive 10 mM methionine in the absence (CONTROL) or presence of 5 μM FCCP or 10 μM diuron. Incubation was continued at 3000 lux for 0, 1, 3, 7, and 13 hr of chase. At the given times, samples were removed from each dish, cell membranes were isolated, and the associated proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (125,000 cpm of hot trichloracetic acid-precipitable radioactivity per sample) as described in the legend to Fig. 1. The positions of the 32-kDa protein and LHC protein (LHCP) are indicated.

plants, it was important to ascertain whether, under *in vivo* conditions, FCCP and diuron act to inhibit ATP formation and electron transport, respectively. As reported in Table 1,

Table 1. *In vivo* effects of the uncoupler FCCP and electron transport inhibitors on *Spirodela* plants

Treatment	ATP content* nmol/mg of chlorophyll	ATP content* %	O_2 evolution† RPS	O_2 evolution† %
None (light control)	925	100	2.95	100
Darkness	650	70	0	0
Diuron, 10 μM	1600	172	0	0
FCCP, 5 μM	325, 160‡	35, 17‡	1.67	57
FCCP, 10 μM	385	42	0.28	9
DBMIB, 5 μM	970	105	2.60	88
DNP-INT, 2.5 μM	830	90	2.41	82

DBMIB, dibromomethylisopropylbenzoquinone; DNP-INT, dinitrophenyl ether of iodonitrothymol.

*Steady-state light-grown *Spirodela* plants (2000 lux) were transferred to separate Petri plates with fresh mineral medium and incubated overnight at 3000 lux at 25°C. The indicated treatments were given then and the plants were incubated for an additional 2 hr in the light or in darkness as listed. Plants were homogenized in ice-cold 0.125 M perchloric acid, and the homogenate was left in the dark on ice for 1 min and then neutralized at 25°C with 0.5 M KOH. The insoluble residue was removed by centrifugation at 5°C and the supernatant was assayed for ATP content (28).

†Oxygen evolution was measured in intact fronds infiltrated with test compounds for 1 hr prior to measurement. RPS (relative photoacoustic signal) was determined as described (29).

‡Values are for plants incubated 7 hr.

5 μM FCCP reduced the total cellular ATP content below that of the dark control while affecting oxygen evolution to a much lesser extent. However, at 10 μM FCCP, a concentration known to affect electron transport in isolated thylakoid systems (39), oxygen evolution was severely inhibited *in vivo* as well. Thus, under the conditions of the experiment shown in Fig. 5, the major effect of FCCP was in uncoupling phosphorylation and not on electron flow. On the other hand, incubation with diuron did not reduce ATP content *in vivo* [if anything, it stimulated it (cf. ref. 40)], although oxygen evolution was abolished. Thus, this herbicide acts *in vivo* in *Spirodela* to inhibit electron transport, a conclusion arrived at in intact algal cells as well (41).

It was of interest to determine if other inhibitors of electron flow, whose sites of action were defined by using isolated thylakoids, were active in intact *Spirodela* plants in an analogous manner. In particular, dibromomethylisopropylbenzoquinone and dinitrophenyl ether of iodonitrothymol, compounds affecting electron flow in isolated thylakoids at a site between plastoquinone and plastocyanin (22), were tested. Table 1 shows that oxygen evolution *in vivo* was not markedly inhibited in their presence. Thus, a more detailed analysis of the segment of the electron transport chain involved in regulating catabolism of the 32-kDa protein *in vivo* is not yet possible with this system.

DISCUSSION

Regulatory Scheme for Metabolism of the 32-kDa Protein. A scheme describing regulation of metabolism of the 32-kDa protein in *Spirodela* and its relationship to photosynthetic electron transport is presented in Fig. 6. We postulate that light controls synthesis of the 32-kDa protein through photophosphorylation. In this regard, recent findings indicate that light acts on synthesis of the 32-kDa protein at a post-transcriptional level in the mature chloroplast (unpublished data). Previous studies comparing etiolated and green tissue (2, 5, 34) did not distinguish between specific regulation by light and chloroplast development per se. Light also controls degradation of the 32-kDa protein, in this case, through photosynthetic electron flow. Thus, via different mechanisms, both the anabolic and catabolic steps are photoregulated. Dual regulation of protein metabolism by a specific, external stimulus has also been noted in other systems. For example,

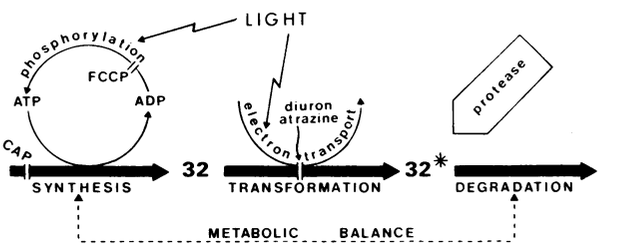

FIG. 6. Regulation of 32-kDa (designated 32) protein metabolism by light. All of the events occur on the thylakoid membranes of the chloroplast. Synthesis is dependent upon ATP derived from photophosphorylation. Inhibition can be achieved either by uncoupling phosphorylation (with FCCP) or by interfering with translation on the chloroplast ribosome [with D-*threo*-chloramphenicol (CAP) (33, 34)]. Transformation occurs during light-driven electron flow through the photosystems (13). The molecular change(s) may involve redox events, substrate binding, or other conformational rearrangements (42). As a result of these changes, the transformed protein (designated 32*) is prone to degradation. The herbicides diuron and atrazine inhibit both electron flow through photosystem II (13, 22, 23) and the susceptibility of the 32-kDa protein to degradation. Degradation of the transformed protein (32*) is catalyzed by a membrane-bound protease. Metabolic balance for the 32-kDa protein is normally maintained over a wide range of light intensities and in the dark (when both synthesis and degradation are minimal).

the role of oxygen in inducing cytochrome P-450 synthesis and degradation in yeast has been reported (43).

The 32-kDa protein is involved in electron flow at the reducing side of photosystem II (13, 14). We postulate that, as a consequence of this role, the protein becomes prone to degradation. Indeed, in isolated thylakoids, the 32-kDa protein has been shown to undergo light-dependent transformation that affects its susceptibility to trypsin (13). Recent experiments indicate that the activity responsible for *in vivo* degradation of the 32-kDa protein is bound to the photosynthetic membrane (unpublished data). Endoprotease activities having a high affinity for denatured protein substrates have been noted previously in the thylakoids (44). Finally, we note that under a very wide range of light conditions, an apparent balance in the rates of synthesis and degradation of the 32-kDa protein is normally maintained *in vivo*. As such, we anticipate finding a steady, low level of this protein in matured plants throughout the diurnal cycle.

Results of fluorescence induction experiments, carried out with intact *Spirodela* plants (34), can be interpreted as showing that partial depletion of the 32-kDa protein from the photosynthetic membrane directly affects the photochemistry at the reducing side of photosystem II (45). This would support theories (42) regarding this protein as a functional component of the secondary acceptor of photosystem II. On the other hand, maintenance of the low, steady-state level of the 32-kDa protein in the light (24, 25, 46, 47), in spite of its massive synthesis, represents a programming of gene expression designed for regulation (48). Expression of such regulatory genes is characterized by high energy costs, which purchase an ability to respond quickly, dramatically, and temporarily to perturbations in the steady state. Such features are characteristic of the 32-kDa protein. Thus, further experimentation will be required to decipher the exact role of this protein in photosynthetic electron transport.

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