Hypothesis on the Control of D1 Protein Turnover by Nuclear Coded Proteins in *Chlamydomonas reinhardtii*

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A hypothesis is presented on the events in the degradation of the D1 protein of photosystem II in the light. It proposes the existence of a nuclear encoded cleavage system that is turning over and which is modulated by its phosphorylation state. A new experimental approach is presented in which the D1 protein degradation under photoinhibitory light is tested in *Chlamydomonas reinhardtii* grown under phosphate deficiency and pretreated with cycloheximide.

Under these conditions the degradation of the D1 protein is delayed whereas in *Chlamydomonas reinhardtii* grown in full medium the D1 protein is rapidly disappearing in high light upon addition of chloramphenicol (CAP) or lincomycin for inhibiting the resynthesis of the D1 protein . Cycloheximide (CHI) has little effect on photoinhibition in such control cells. In cells grown, however, for 20 h in phosphate deficiency – such that there is not yet loss of photosynthesis capacity – pretreatment with cycloheximide or canavanine in low light the degradation of the D1 protein even in 6 h high light is prevented to an appreciable extent. Further addition of CAP or lincomycin has only a small effect. [^{14}C]leucine incorporation was used to show that there is no resynthesis and that the presence of the D1 protein is due to a delay of degradation.

The results are interpreted to show that excess high light which converts the D1 protein into a potentially, degradable mode is not sufficient for degradation of the D1 protein. A cleavage system is needed as well. It is postulated that under phosphate deficiency and pretreatment with CHI or canavanine a nuclear coded cleavage system for the D1 protein is depleted, *i.e.* the cleavage system for the rapidly turning over D1 is also turning over.

It is shown that under phosphate deficiency an alkaline phosphatase activity in the chloroplast and the thylakoid membrane of *Chlamydomonas reinhardtii* is increased. It is proposed that the ratio of kinase/phosphatase converts an active, stable phosphorylated cleavage system into a labile unphosphorylated and turning over state.

Introduction

Exposure of *Chlamydomonas reinhardtii* cells to high light intensities leads to loss of photosynthesis capacity and degradation of one of the reaction center polypeptides of PS II, the D1 protein. This loss may be compensated by resynthesis of the D1 protein. Photoinhibition becomes apparent when resynthesis of the plastome coded D1 protein is prevented by addition of chloramphenicol or lincomycin. This process has been well studied in *Chlamydomonas reinhardtii* particularly by Ohad and his collaborators (Prásil *et al.,* 1992) and others (see references in Prásil *et al.,* 1992). The mechanism of photoinactivation of PS II followed by degradation of the D1 protein has been studied extensively also in isolated thylakoids for several decades. Recently detailed proposals for the response of the reaction center of PS II and for cleavage sites in the D1 protein were reported in particular from Barber and Andersson (1992) in so-called donor and acceptor site inhibition respectively. The related phenomenon of the rapid turnover of the D1 protein is also long known and is studied in particular in *Spirodela* by Edelman and his collaborators (for review see Mattoo *et al.,* 1989). This group closed in on the primary cleavage site in the amino acid sequence of the D1 protein in rapid turnover to be located in an extended loop between the 4th transmembrane helix D and a parallel helix DE (Greenberg *et al.,* 1987). Recently a model for the control of the accessibility

Abbreviations: CAP, chloramphenicol; CHI, cycloheximide; LHCP, light-harvesting chlorophyll protein; Q_B, second plastoquinone-binding site on photosystem II; PQ, plastoquinone; PS II, photosystem II.

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of the extended loop (Trebst *et al.*, 1988) with the cleavage site in rapid turnover has been proposed on the basis of the occupancy state of the Q_B site in the D1 protein by quinone or specific inhibitors (Jansen *et al.*, 1993). Accordingly, the relaxed state of the extended loop is cleaved whereas in a state arrested by plastoquinone or by certain inhibitors the D1 protein is stable.

The assumed proteolytic system that cleaves the D1 protein in rapid turnover has not been identified. Sometimes even doubts have been raised that there exists one, *i.e.* chemical cleavage of the protein by radicals generated by triplett states of the reaction center with oxygen or autocatalytic cleavage. In photoinhibition the 43 kDa protein of PS II in thylakoids is suspected by Salter *et al.* (1992) to carry a serine type proteolytic activity.

Here we present evidence for a proteolytic system *in vivo*. We conclude this from the observation that the degradation of the D1 protein and the photoinhibition of PS II caused by several hours of high light in *Chlamydomonas reinhardtii* cells can be blocked by pretreatment with inhibitors of the translation of nuclear encoded proteins. The necessity of prior phosphate deficiency of the cell for stability of the D1 protein is interpreted to indicate that the putative cleavage system is under control of a kinase/phosphate system. The inactive, dephosphorylated cleavage system is turning over and is depleted when phosphatases have been induced by phosphate deficiency and CHI prevents resynthesis.

Materials and Methods

Chlamydomonas reinhardtii was grown in TAP (Tris/acetate/phosphate) medium containing 20 mM Tris, 1 mM KH_2PO_4/K_2HPO_4, pH 7.2, 7.5 mM NH_4Cl, 0.35 mM $CaCl_2$, 0.4 mM SO_4, 0.1% (v/v) microelements according to Amrhein and Fillner (1973) and at a light intensity of 70 W/m^2 at 20 °C, bubbled with 5% CO_2/air. After 24 h growth an aliquot of the cells were resuspended either in full or in phosphate-deficient medium. After additional 20 or 24 h growth in low light (70 W/m^2) the inhibitor protocol described in Table I and the figures was started. The phosphate-deficient cells were grown in the same medium, except that phosphate was left out and 1 mM KCl added. For high light the cell cultures were exposed in the same medium to 2000 W/m^2 for the time given in Results.

Thylakoid membranes from the *Chlamydomonas reinhardtii* cells were prepared by sonication (4×15 s) and differential centrifugation. Photosynthetic activity was followed spectroscopically of ferricyanide reduction at 420 nm plus or minus a lipophilic quinone mediator.

Intact chloroplasts were prepared according to Harris (1989). We thank S. Schrader, Bochum, for providing the autolysin. Immunoblotting of the D1 protein was done with an antibody against a truncated D1 protein as of amino acid 161. The values in Table I for immunoblots and autoradiographs are obtained by laser screening. Phosphatase activity was measured by following the hydrolysis of *p*-nitrophenylphosphate at 400 nm at pH 8.

Results

Under photoinhibitory conditions (*i.e.* strong light of 30 times 70 W/m^2 light) the D1 protein of photosystem II is photoinactivated and then degraded (see Prásil *et al.*, 1992; Andersson and Barber, 1992). Resynthesis of D1 protein may mask this by reassembling active PS II. Therefore often CAP is added to the experiment to prevent any resynthesis (see Prásil *et al.*, 1992). This principal property of D1 degradation in photoinhibition is again seen in the control experiment in Fig. 1. Photosynthetic activity continues after an acetate grown *Chlamydomonas reinhardtii* cell suspension is brought into strong light (2000 W/m^2) after 24 h with 70 W/m^2 light. Both addition of 0.6 mM CAP or 1 mM of the arginine antagonist canavanine lead to a rapid disappearance of photosystem II activity and of the D1 protein measured in isolated thylakoids prepared from such cells after 2 or more hours in high light. Addition of canavanine and then later also CAP after 2 or 4 h high light completely abolishes activity.

These known properties of photoinhibition were markedly changed when *Chlamydomonas reinhardtii* was grown under phosphate deficiency for 24 h. As Fig. 2 shows, addition of 0.6 mM CAP still markedly reduces photosynthetic activity though not to zero activity. But 1 mM canavanine is no longer an effective inhibitor and CAP added after canavanine pretreatment no longer abolishes ac-

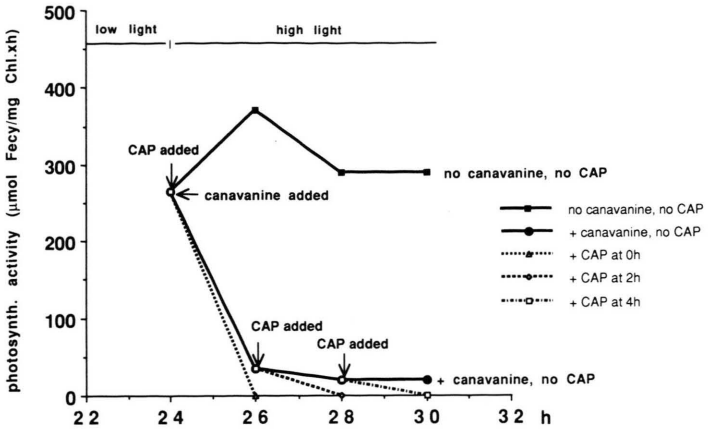


Fig. 1. Effect of canavanine and CAP in photoinhibition in *Chlamydomonas reinhardtii* grown in full medium. After growth for 24 h in low light, high light was turned on ±1 mM canavanine. In the canavanine samples 0.6 mM CAP were added at the time indicated by the arrows (*i.e.* 0, 2 and 4 h high light).

tivity. The later CAP is added, the smaller is its effect. As shown below in Table I, there is no [^{14}C]leucine incorporated under any conditions in the presence of CAP – as expected – *i.e.* the activity of photosystem II is not due to newly synthesized proteins. Lincomycin (0.45 mM) has – though somewhat less pronounced – the same effect as CAP. The immunoblot in Fig. 3 A shows that indeed in cells grown in phosphate medium canavanine and lincomycin lead practically to a disappearance of the D1 protein whereas in phosphate-deficient cells there is an appreciable amount left. Further documentation is given in Table I.

Fig. 3 B shows the autoradiograph of the thylakoid proteins that became labelled with [^{14}C]leucine. There is incorporation into the D1 protein in both normal and phosphate-deficient cells but none in either culture in the presence of CAP and canavanine. A comparison of these lanes with those of the immunoblot shows that the D1 protein observed in phosphate-deficient cells plus CAP is not due to newly synthesized protein.

Table I compares photosynthetic activity, D1 protein content and [^{14}C]leucine incorporation into the D1 protein in phosphate-containing and phosphate-deficient *Chlamydomonas reinhardtii* cells after 6 h high light. Inhibitors of translation were added after certain times. A typical protocol is shown below in Table I. After 20 h phosphate deficiency, 0.35 mM CHI was added in weak light. Strong light was turned on after 2 more hours in weak light and then canavanine was added. The cells were kept in high light for 4 h and then 0.6 mM CAP and [^{14}C]leucine were added and strong light continued for two more hours. After

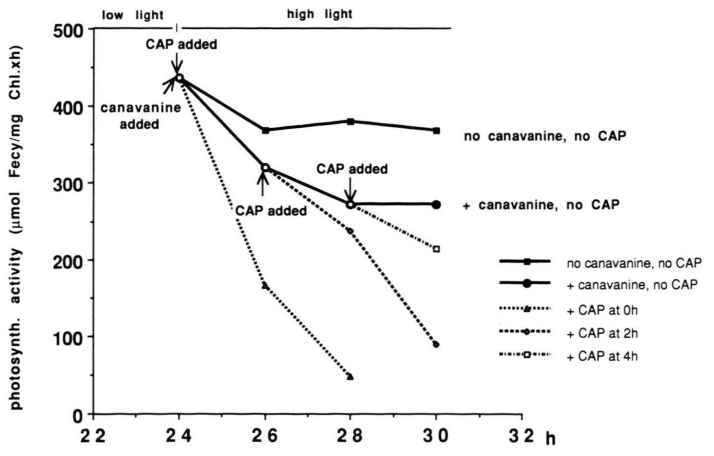
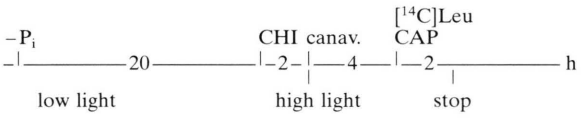


Fig. 2. Effect of canavanine and CAP in photoinhibition in *Chlamydomonas reinhardtii* grown in phosphate-deficient medium. Conditions as in Fig. 1.

Table I. Effect of translation inhibitors on D1 protein turnover. Change in activity and D1 protein of *Chlamydomonas reinhardtii* thylakoids after 6 h high light.

	PS II activity	D1 protein Immuno-blot	D1 protein [^{14}C]Leu incorporation
Phosphate in the medium			
Control	=100	=100	=100
+0.6 mM CAP	0	0	0
+0.35 mM CHI	50	60	20
+1 mM canavanine	10	50	20
+CHI + CAP	0	0	0
+Canavanine + CAP	0	0	<3
No phosphate in the medium			
Control	100	100	40
+0.6 mM CAP	10	10	0
+0.35 mM CHI	70	80	20
+1 mM canavanine	70	140	5
+CHI + CAP	90	40	0
+Canavanine + CAP	60	40	<1

Time protocol of the experiment in hours: after 20 h growth ± phosphate in low light, 0.35 mM CHI were added. After two more h in the low light 1 mM canavanine was added and high light turned on. After 4 h high light 0.6 mM CAP and [^{14}]leucine were added and high light continued. After 2 h light was turned off, the cells centrifugated and thylakoids prepared and measured as described in Fig. 3.



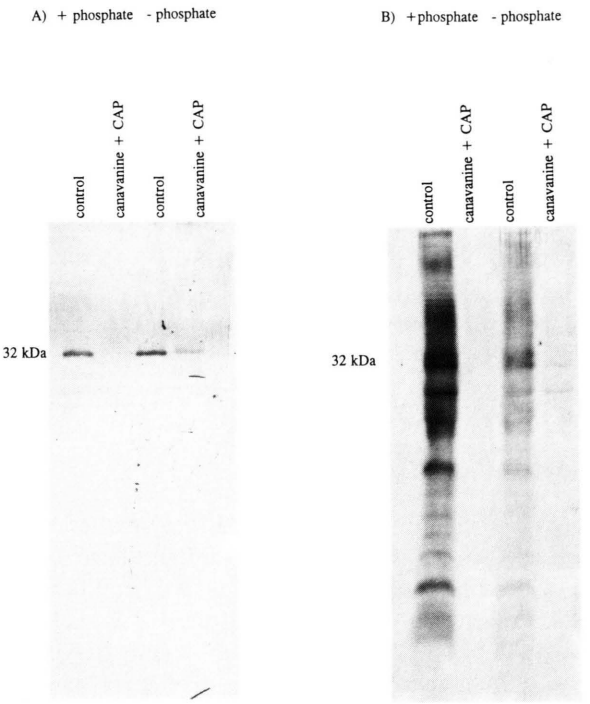


Fig. 3. Effect of translation inhibitors on the stability and synthesis of the D1 protein in control and phosphate-deficient *Chlamydomonas reinhardtii*. A) Immunoblot of the D1 protein after 4 h photoinhibition. B) Autoradiographic thylakoids after incubation of the cells with [^{14}C]leucine. A and B are the same sample in which *Ch. reinhardtii* cells grown for 20 h plus or minus phosphate. High light was then turned on for 2 h. 1 mM canavanine plus 0.6 mM CAP were added at the beginning of high light.

the light was turned off, thylakoids were isolated and the three parameters measured. Pretreatment with CHI diminishes activity of the D1 protein content in phosphate-deficient cells somewhat less than in phosphate-grown cells (after 4 h strong light only there is no effect of CHI in phosphate medium and even stimulation in phosphate-deficient cells; data not shown here but given in the Thesis of E. Bracht, 1994). CAP completely abolishes PS II activity and leads to complete degradation of the D1 protein in phosphate medium but does not completely in phosphate-deficient cells – see also Fig. 1 and 2. The effects of canavanine are quite different in the two types of cells. Canavanine inhibits photosynthesis in phosphate-grown cells but it does not in phosphate-deficient grown cells. If both types of translational inhibitors are added – see detailed protocol shown below in Table I – *i.e.* canavanine or CHI plus CAP there is a marked difference between plus and minus phosphate-grown cells. In phosphate-grown cells there is no PS II activity and no D1 protein left. In phosphate-depleted cells, even in high light conditions, the D1 protein is not completely degraded and an appreciable photosynthetic activity remains. [^{14}C-]Leucine incorporation was used to check for absence or presence of protein synthesis. There is no [^{14}C]leucine incorporation into the D1 protein band in the presence of CAP in cells grown in the presence or absence of phosphate, see also Fig. 3. Therefore the D1 protein observed in the cells grown without phosphates in the presence of CAP by immunoblotting represents D1 protein which had not been degraded in the cells grown without phosphates as opposed to phosphate-containing cells. Photosynthetic activity is – as expected – more sensitive to high light due to

photoinactivation preceding D1 degradation. There is photoinactivation in the control as well as in the deficient cells for example when activity appears to be higher than the D1 protein, this is due to photoinactivation in the control, because the values are given in percent of the control.

Similar data of the kind in Table I are given in Fig. 1 and 2. Actually the values in the Fig. 1 and 2 after turning on high light, are computed from those in Table I. The figures show more clearly that in phosphate-depleted cells the addition of CAP does not abolish photosynthesis activity after pretreatment with canavanine and that the extent of stabilization depends on the time after which CAP was added. The later CAP is added the higher is the activity remaining – due to an increased stability of the D1 protein – some of which had been resynthesized during the period before CAP addition. And *vice versa* – as discussed below – the later the cleavage system had been depleted. Instead of 1 mM canavanine we used 0.35 mM CHI (no further data than those in Table I are given here but in the Thesis of E. Bracht, 1994).

The immunoblots of the D1 protein (Fig. 3A) and the autoradiographs of leucine incorporation (Fig. 3B) into the D1 protein are given in Table I for some of the points in the Fig. 1 and 2.

Phosphate-deficient cells of *Chlamydomonas reinhardtii* have further changed properties in addition to the stabilization of the D1 protein under high light. A major change in phosphate deficiency is the induction of phosphatases. This is known already for phosphatases in the outer membrane (Dumont *et al.*, 1993). As Table II shows there is also an induction of alkaline phosphatase activity in the membrane fraction and in the "broken cell supernatant" fraction measured after sonicating cells. Also in the matrix (stroma) compartment of the chloroplast and in the thylakoid membranes phosphatase activity is increased. As this membrane fraction is not pure, intact chloroplast were first isolated by autolysin treatment and then osmotically broken and separated by centrifugation according to Harris (1989). The phosphatase activity in the membrane fraction appears to be peripherally attached as it can be increased by mild detergent treatment (Table II). We have not yet studied these membrane-bound phosphatases activity in detail. Octylglucoside treatment may not be the optimal way to release the bound phosphatase. We have preliminary results that the soluble phosphatase in the chloroplast is fluoride-sensitive (but not 100%). This has to be studied in more detail. The pH optimum is at pH 8, *i.e.* they are alkaline phosphatases. An alkaline phosphatase has recently been purified by Kieleczawa *et al.* (1992), thought to be the phosphatase involved in LHCP dephosphorylation. Indication for a light-dependent phosphatase for D1 protein dephosphorylation was recently given by Elich *et al.* (1993). We have not yet tested protein phosphatases.

Table II. Increase of alkaline phosphatase activity – either exported or inside the chloroplast – in *Chlamydomonas reinhardtii* grown in phosphate deficiency. Phosphatase activity is given in μmol nitrophenylphosphate hydrolyzed/15 min/ml wet cells contained 40 μmol chlorophyll/ml.

	Phosphatase activity in (in the growth medium)	
	+Phosphate	No phosphate
From 50 ml wet cells:		
Growth medium	0	80
Washed cells	0	38
Membrane fraction (after sonication of cells)	2.3	7.7
Membrane fraction +0.1% octylglucoside	4.1	16.2
Broken cell supernatant (after sonication of the cells)	11.4	30
From isolated chloroplasts:		
Thylakoid membranes	1.7	2.8
Membranes +0.1% octylglucoside	3.4	4.6
Stroma fraction	3.2	4.4

Discussion

The proteolytic system that cleaves the D1 protein in rapid turnover or in photoinhibition after light has moved the protein in an accessible mode (Jansen *et al.*, 1993) has not been identified. Salter *et al.* (1992) have suggested on the basis of inhibitor studies in isolated thylakoids that it may be the chloroplast coded 43 kDa antenna protein of PS II that has this proteolytic activity on the basis of labelling of this protein by diisopropyl-fluorophosphate which inhibits D1 degradation in their system. Autocatalytic cleavage of the D1 protein is discussed by Komenda and Barber (1994). We report here on conditions in which the D1 protein

in *Chlamydomonas reinhardtii* is stable even under high light.

The protocol for stabilization of the D1 protein and PS II activity is that the cells were grown under phosphate deficiency where likely phosphatases have been induced. If the cells were then (pre-)treated with inhibitors of nuclear coded proteins – CHI or canavanine – the further addition of CAP or lincomycin did not change the D1 protein content and therefore excluded that the measured D1 protein and the PS II activity are due to resynthesis of the protein and repair of PS II. It is directly shown that under these conditions indeed [^{14}C]leucine is not incorporated into the D1 protein.

The results in this paper are published in detail in the Thesis of E. Bracht (1994) indicate a new approach for clarifying the reaction sequence in rapid turnover of the D1 protein. They show that not only the D1 protein has to be brought into an accessible state for degradation as already discussed by Jansen *et al.* (1993), but that also a cleavage system exists that is under light control as well.

We like to propose a hypothesis for the control of PS II activity and of D1 protein degradation and for the regulation of the cleavage system from an interpretation of our results (Fig. 4).

A nuclear coded cleavage system for the D1 protein was shown to exist here, *i.e.* the degradation is not an autocatalytic process induced by oxygen radicals. The cleavage system exists in an active and an inactive state depending whether it is phosphorylated or not. A kinase activity may be light-controlled possibly by the PQ/PQH$_2$ state. From the mutant studies of Gong and Ohad (1991) (see below) we like to suggest that the redox state of plastoquinone for activation of the kinase is oxidized. The unphosphorylated cleavage system is inactive, labile, and turning over. We assume that under phosphate deficiency phosphatases are induced. They will change the ratio of kinase/phosphatase activity towards the dephosphorylation state and the cleavage system is brought into the labile state. The turnover rate of the dephosphorylated cleavage system may get almost close to that of the D1 protein itself. Preventing resynthesis of the protein(s) of the cleavage system by inhibition of translation, *i.e.* by CHI or canavanine, eventually causes its depletion. Therefore after 6 h high light we find a much retarded degradation of the D1 protein in phosphate-deficient *Chlamydomonas reinhardtii* cells. This hypothesis may indicate that the D1 protein controls the phosphorylation of its cleavage system by stabilizing or

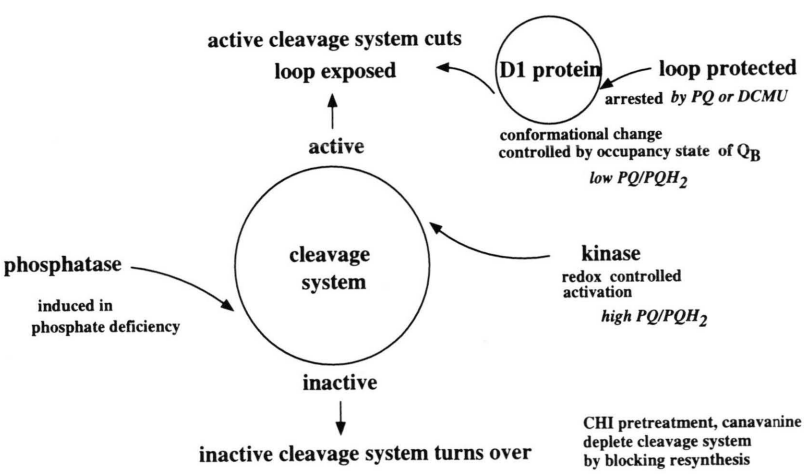


Fig. 4. Schematic representation of the hypothesis for the existence, role and control of a cleavage system in the degradation of the D1 protein. For degradation to occur both the substrate – the D1 protein – and the enzyme have to be poised. A specific conformational change exposes the extended loop (AS 230–247) of the D1 protein (the "exposed state"). The loop is in the "arrested state" when the Q_B site is occupied. The cleavage system cuts in the exposed loop when it is phosphorylated by a redox-controlled kinase and thus converted to the active state. Phosphatases – in the experiment described induced by phosphate deficiency of the cell – dephosphorylate the cleavage system to an inactive state. As the cleavage system turns over, CHI pretreatment depletes it by preventing resynthesis.

destabilizing it and this way may induce its own degradation. As the cleavage system is turning over the protein(s) might therefore not necessarily be present in PS II preparations, where one would look for identification of this protease. The cleavage system may also contain a chloroplast-coded component *i.e.* a two subunit system like the Clp protease system described by Maurizi *et al.* (1990). This chloroplast coded subunit of the cleavage system maybe indeed be indicated by recent results of Kim *et al.* (1993) who find a stable D1 protein aggregate in high light grown *Dunaliella* in the presence of CAP. The Clp system is ATP-dependent and such a system was shown to exist in the chloroplast by Liu and Jagendorf (1984). ATP deficiency, though, is not likely to be the cause of stabilization of the D1 protein in our experiments. Note that phosphate-deficient cells grow autotrophically. We call the protein-degradating activity a cleavage system rather than a protease at this time because light and oxygen requirement for D1 degradation, as is usually assumed (see Prásil *et al.*, 1992; Barber and Andersson, 1992) may suggest that it is an oxidase rather than a protease; but this is not entirely clear yet.

The D1 protein after 6 h high light under the protecting conditions is partly photosynthetically active. This may indicate that also active D1 protein can be degraded, *i.e.* it does not necessarily require prior photoinactivation of the D1 protein. This means that we observe rapid turnover in high light like that in *Spirodela* (Greenberg *et al.*, 1987), but not necessarily photoinhibition.

In addition to a proper poise of the cleavage system, also the substrate – the D1 protein – has to be in an accessible state in order for degradation to be possible (Jansen *et al.*, 1993). This is also a redox-controlled process *via* the redox and occupancy state of Q_B but likely in a way opposite to the poising of the cleavage system. We proposed that the occupancy state of the Q_B controls the cleavage site in the extended loop in the amino acid sequence of the D1 protein by changing its accessibility to proteolytic activities (Trebst *et al.*, 1988; Jansen *et al., 1993). In a relaxed and accessible state of the loop the* Q_B site is empty. The loop is arrested and stabilized when plastoquinone or certain inhibitors occupy the site. This concept has recently been substantiated by comparing the correlation of properties of rapid turnover in *Spirodela* with the properties of trypsin sensitivity at arg 238 – a site also in the extended loop – and with the response to mutations in the Q_B site towards phenolic inhibitors with specific side chains (Jansen *et al.*, 1993). These correlations show a real specific change in accessibility of specified amino acid. The cleavage site in the extended loop of D1 is in a contact site between the extended loop of both the D1 and D2 proteins (Trebst, 1991), and it is this contact that is loosening up in the conformational change. The cleavage is therefore specific for a position in a domain or area rather than specific for a particular amino acid as mutant studies have recently shown by Kless *et al.* (1992). These studies also show that these two conformational states of the D1 protein are photosynthetically active.

The concept of an empty or reduced Q_B site for getting the D1 protein into a degradable state (relaxed mode of the loop) appears to be accepted and is supported by the studies with thylakoids by Andersson and Styring (1991). It is, however, challenged by Prásil *et al.* (1992) and Gong and Ohad (1991), who suggest that – just the opposite – an oxidized Q_B site poises the D1 protein for degradation. This they concluded from photoinhibition studies with *Chlamydomonas reinhardtii* mutants with deletion of either the cytochrome b_6/f complex, plastocyanin or PS I in which therefore the PQ pool remains reduced (Gong and Ohad, 1991). In such systems the D1 protein is not degraded. We propose here that these results of Gong and Ohad do not show the properties how to get the D1 protein poised for the degradation but rather that they observe the control of the cleavage system by the redox state of plastoquinone. We propose that in Ohad's mutant experiments the cleavage system is inactive when plastoquinone is reduced. This predicts that when weak light slowly oxidizes PQH_2 after that state in high light the cleavage system is reactivated and then the D1 protein is degraded. This has indeed recently been observed by Zer *et al.* (1994).

There is another aspect of the recognition of a certain part of the D1 protein by another protein. The concept of a conformational change of the contact site of the two loops of the D1 protein by the redox state of Q_B is also possibly the mechanism for communication of the Q_B site *via* the loop to redox-controlled activation of other regu-

latory proteins, like kinases, translational cofactors or DNA-binding proteins that sense the conformational change in the loop. The significance of redox-controlled gene expression has recently been explored by Allen (1993).

Acknowledgements

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