Light-dependent degradation of the Q_B-protein in isolated pea thylakoids

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The 32 000-dalton Q_B-protein of photosystem II (PS II) is rapidly damaged and removed from isolated pea thylakoids during incubation in the light resulting in a loss of photosynthetic electron flow through PS II. This *in vitro* photo-inhibition is similar to that previously reported with intact *Chlamydomonas* cells. The damage occurs at a faster rate *in vitro*, however, due to the inability of isolated thylakoids to synthesize replacement Q_B-protein. The removal of the damaged Q_B-protein does not require any soluble components of the chloroplast stroma and is unaffected by the protease inhibitors phenyl-methylsulfonylfluoride or antipain. Unlike the effect of trypsin, no low mol. wt. membrane-bound or soluble fragments of the labelled Q_B-protein could be identified either by autoradiography or immunologically using polyclonal antibodies specific for the Q_B-protein. The light-induced damage to the Q_B-protein (indicated by a loss of Q_B functional activity), preceded the removal of the protein from the membrane. We conclude that photodamage of the Q_B-protein generates a conformational change which renders the protein susceptible to attack by a highly efficient, intrinsic membrane protease.

Key words: herbicide/photosystem II/protein turnover/quinone-binding/32 kd

Introduction

The light-induced turnover of the 32 000-dalton polypeptide of photosystem II (PS II) has been studied *in vivo* using *Spirodella* (Mattoo *et al.*, 1981), *Chlamydomonas* (Wettern and Ohad, 1984) and several higher plants (Hoffman-Falk *et al.*, 1983). This protein has recently been shown to be equivalent to the Q_B-protein (Ohad *et al.*, 1984), and its damage is reflected in a loss of PS II activity at the Q_B site (Kyle *et al.*, 1984a). Arntzen *et al.* (1984) have proposed that damage to the Q_B-protein occurs as a consequence of its enzymatic function, which involves the stabilization of reactive quinone anion radicals. Furthermore, they suggested that the physiological phenomenon of photo-inhibition *in vivo*, or light-induced loss of PS II activity occurs when the rate of damage to the Q_B-protein exceeds the rate of its *de novo* synthesis and insertion into the functional PS II units (for review, see Kyle and Ohad, 1984).

The turnover rate of the Q_B-protein has been estimated to be considerably faster than the turnover rates of any other chloroplast protein (Mattoo *et al.*, 1981). This implies a very specific and efficient mechanism for identifying and removing the damaged protein. Here we present the first description of the damage and removal of the Q_B-protein of a higher plant *in vitro*. Furthermore, we have utilized this *in vitro* system for a preliminary

characterization of the properties of this highly efficient membrane protein degradation activity.

Results

Labeling of cut pea seedlings with ³⁵SO₄ for short periods (<4 h) leads to the rather specific labeling of a polypeptide (mol. wt. 32 000) running somewhat slower in lithium dodecylsulfate (LDS)-PAGE than the predominant thylakoid membrane proteins of the light-harvesting complex (Figure 1). Labeling experiments

Fig. 1. Identification of the rapidly turning over 32 000-dalton polypeptide as the Q_B-protein. The Coomassie-stained gel and autoradiogram are shown for thylakoid polypeptides prepared from plants pre-labelled for 1 h with ³⁵SO₄ (1 mCi/μmol), and for 4 h with [¹⁴C]lysine (330 mCi/mmol), [¹⁴C]-arginine (336 mCi/mmol) or [³⁵S]methionine (1 mCi/μmol). The Q_B-protein (Q_B), the 43-kd PS II polypeptide (☆), and the light-harvesting complex (LHC) used as the internal standard for measuring specific protein turnover are indicated.

Fig. 2 Kinetics of PS II inactivation (a) and protein turnover (b) *in vitro*. The rates of reduction of silicomolybdate ($H_2O \rightarrow SiMo$, independent of Q_B) and dichlorophenol-indophenol ($H_2O \rightarrow DPIP$, dependent on Q_B) are the means of three replicates and are shown as percent of control (not photo-inhibited) values. Control rates $H_2O \rightarrow SiMo = 80 \mu E/mg \text{ Chl/h}$; $H_2O \rightarrow DPIP = 160 \mu E/mg \text{ Chl/h}$. Protein turnover values for the Q_B -protein (32 kD) and a PS II reaction center protein (42 kD) were obtained from densitometric scans of autoradiograms by calculating the ratio of the peak areas of each specific band relative to that of LHC. Values shown are percentages of the control (not photo-inhibited) samples.

for longer time periods (4 h) using [^{14}C]arginine or [^{14}C]lysine indicate a lack of ^{14}C incorporation into this band when [^{14}C]lysine was used as a precursor. The chloroplast gene *psbA* from different higher plant species, which codes for the Q_B -protein, has been cloned, and its nucleotide sequence has been determined (for review, see Kyle, 1984). Based on its amino acid sequence, inferred from the sequence of the *psbA* gene, it appears that the protein does not contain the amino acid lysine. Consequently, the lack of labeling by lysine can be used as a marker for the Q_B -protein. On the other hand, [^{35}S]methionine provides a good labeling of the Q_B -protein (Figure 1) and was used routinely for the turnover experiments.

Instability of PS II activity of isolated thylakoids in the light, especially at dilute chlorophyll concentrations, is a common observation. If this instability is due to photo-inhibition as recently described by Kyle *et al.* (1984a,b), then the first component to be damaged should be the Q_B -protein. Indeed, the light-induced loss of Q_B -dependent PS II activity ($H_2O \rightarrow DPIP$) preceded that of a Q_B -independent PS II activity ($H_2O \rightarrow SiMo$) (Figure 2a). The half-times for the loss of these PS II activities were 10 and 30 min, respectively, under the light intensity and incubation conditions we used.

In parallel with the activity measurements, we quantitated the ^{35}S -radioactivity in the Q_B and the 42-kD polypeptide (thought to be associated with the PS II reaction center, Satoh *et al.*, 1983)

Fig. 3. Photodegradation of Q_B -protein in isolated thylakoids. The thylakoids ($50 \mu g \text{ Chl/ml}$) were illuminated ($1000 \mu E/m^2/s$) for 15, 30, 45 and 60 min, pelleted and the membrane proteins were separated by LSD-PAGE using acrylamide gradients of 8–15%. Mol. wt. standards included bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (29 000) and cytochrome c (12 400 daltons).

Table I. Degradation of the Q_B-protein in the light (1000 $\mu\text{E}/\text{m}^2/\text{s}$; 60 min) or dark with various additions

Treatment	Q _B -protein (% of dark control)
Dark (no additions)	100
+ dithionite (10 mM)	92
Light (no additions)	36
+ ferricyanide (1 mM)	73
+ hydroxylamine (10 mM)	29
+ PMSF (10 mM)	26
+ antipain (2 mg/ml)	33
+ ATP (5 mM)	40
+ ADP (5 mM)	95
+ DCMU (10 μM)	73

relative to that of the major polypeptide of the light-harvesting complex (LHC) at 28 kd (used as an internal standard). The loss of the Q_B and PS II reaction center proteins mimicked the loss of the photochemical activities, but with decay half-times of 17 and 66 min, respectively (Figure 2b). These data show that the loss of activity precedes the loss of the protein from the membrane. Moreover, addition of the chloroplast soluble stroma to the incubation system did not affect the degradation of the Q_B-protein *in vitro* (data not shown).

Using intact *Chlamydomonas* cells, we have previously shown that the loss of radioactively labeled Q_B-protein induced at low light intensities does not parallel the loss of the weakly Coomassie-stained protein band in the thylakoid polypeptide profiles (Wettern and Ohad, 1984). This is due to an ongoing synthesis and replacement of damaged protein with the new Q_B-protein *in vivo*. In isolated thylakoids, however, this is not the case. As photoinhibition proceeds, there is a parallel loss of labeled Q_B-protein and of the diffuse Coomassie-stained band (Figure 3). It is also apparent that during the initial stage of photo-inhibition, there is a rather specific loss of the Q_B-protein. In addition, no low mol. wt. labeled fragments were visible on the autoradiogram at any stage of photo-inhibition. However, the radioactivity in the supernatant (following pelleting of the thylakoids) increased, indicating that the degradation products were in the soluble fraction. Similar results have been obtained using isolated *Chlamydomonas* thylakoids (Wettern and Ohad, 1984).

We have previously suggested that the Q_B-protein damage occurs as a natural consequence of its enzymatic function which involves the stabilization of quinone anion radicals (Kyle *et al.*, 1984a,b). Furthermore, if regeneration of the terminal acceptor was prevented by blocking electron transfer past the plastoquinone pool, photo-inhibition followed at an increased rate (Kyle *et al.*, 1984a,b). As no terminal electron acceptor was present in the standard incubation mixture with isolated thylakoids, a rapid and extensive photo-inhibition was observed. However, when an acceptor of electrons was added (ferricyanide), the degree of damage was significantly reduced (Table I). Addition of a PS II donor (hydroxylamine), slightly increased the amount of damage, as assayed by Q_B-protein removal (Table I). The reduction of the plastoquinone pool does not seem to be sufficient for damage and removal of the Q_B-protein, since incubation of membranes in the dark with dithionite did not result in the loss of radioactivity from the Q_B-protein.

The protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and antipain had no effect on the removal of the Q_B-protein (Table I). This lack of inhibition may simply reflect the inac-

Fig. 4. Comparison of photo-inhibition (PI) and trypsinization (T) of isolated thylakoids to untreated controls (C). Trypsinization was carried out for 20 min at 24°C with TPK-trypsin (Sigma) at 100 $\mu\text{g}/\text{ml}$. Photo-inhibition was carried out for 30 min at 1000 $\mu\text{E}/\text{m}^2/\text{s}$. In both cases, the Chl concentration was 1 mg Chl/ml, the reactions were stopped by the addition of electrophoresis sample buffer, and the total mixture was analyzed by LDS-PAGE using acrylamide gradients of 10–21%. Mol. wts. are given for the principal tryptic fragments.

cessibility of these protease inhibitors to the membrane-bound protease. Addition of ATP to the incubation system had little effect on Q_B-protein removal. However, addition of 5 mM ADP had a significant effect on the prevention of removal of the Q_B-protein (Table I).

Arntzen *et al.* (1984) have previously shown that herbicides which compete with the plastoquinone for the binding site on the Q_B-protein, reduce the extent of photo-inhibition. This is also the case in isolated thylakoids (Table I). Following 60 min of photo-inhibition in the presence of 10 μM Diuron, less than half of the Q_B-protein was lost, as compared with the control incubated in the light in the absence of the inhibitor.

The Q_B-protein has been shown to be trypsin-sensitive (Steinback *et al.*, 1981), and the trypsin sites have been mapped (Marder *et al.*, 1984). Following trypsin treatment of pea thylakoids, major labeled proteolysis products were visible at 23.2, 15.7, 13, and 9 kd (Figure 4). Based on the kinetics of appearance of these labeled products and the disappearance of the Q_B-protein and LHC-labeled bands, as well as on the results obtained by immunoblotting with the 32 kd-specific antibodies, we conclude that the 23.4-kd fragment arises from the LHC polypeptide, and the major product of Q_B digestion in pea thylakoids is the 15.7-kd band. Following light-dependent degradation of the Q_B-protein *in vitro*, no low mol. wt. products were observed (Figure 3). As the loss of radioactivity from the membrane fraction dur-

Fig. 5. Time course of the initial stages of photoinhibition using the total assay mixture and 10–21% acrylamide gradients. The stained polyacrylamide gel, autoradiogram and immunoblot, using antibodies against the 23 kD fragment of the Q_B -protein, are shown for samples incubated under photo-inhibitory conditions ($1000 \mu\text{E}/\text{m}^2/\text{s}$; 24°C) at $1 \text{ mg Chl}/\text{ml}$ for 0, 4, 8, 12 and 25 min. The dark control was wrapped in aluminum foil for the 25 min incubation period. Total incubation mixtures were loaded on gels for LSD-PAGE. The Q_B -protein is marked with a star.

ing photo-inhibition suggested formation of soluble proteolytic fragments, both membrane and supernatant fractions were assayed for low mol. wt. products. In spite of our efforts to capture the low mol. wt. soluble peptide fragments, no labeled proteolytic products of the ^{35}S -labeled Q_B -protein were observed on LDS-polyacrylamide gels (Figure 4). Although immunoblotting gave a positive reaction for the 15.7-kD trypsin fragment, no immunoreactive bands were observed following photo-inhibition (data not shown). Thus, the cleavage fragments were either small enough to diffuse out of the gel during staining/destaining, or migrated toward the anode and were lost during electrophoresis.

It is possible that the initial proteolytic fragments are rapidly degraded and, therefore, no large peptides would remain after a prolonged photo-inhibition treatment. These may be transiently present and observable, however, shortly after the onset of light incubation. A time course of the initial stages of Q_B -protein damage and removal is shown in Figure 5. Once again, both membrane and supernatant fragments were loaded on the gel, and even when very little degradation of the Q_B -protein had occurred, no ^{35}S -labeled fragments or immunopositive reactions could be seen at lower mol. wts.

Discussion

We have presented the first evidence that the 32-kD, Q_B -protein of PS II of the thylakoid of a higher plant undergoes a light-

dependent selective degradation *in vitro* by a mechanism not requiring soluble components from the chloroplast stroma. The turnover is initiated by damage or structural changes of the protein induced by light, and results in the loss of Q_B -dependent PS II electron translation activity. The structural change renders the protein susceptible to attack by a highly efficient membrane-bound protease(s). This proteolysis is insensitive to PMSF and antipain, does not require ATP, and generates soluble cleavage fragments which could not be detected by autoradiography or immunoblotting.

The fact that Q_B -protein damage and degradation occurs in isolated thylakoids in the absence of a terminal acceptor indicates that degradation is not simply dependent on electron flow, as suggested by Mattoo *et al.* (1981). We have previously reported that although Diuron or atrazine (both of which cause oxidation of the plastoquinone pool) reduce the amount of photodamage in intact *Chlamydomonas* cells, an inhibitor such as dibromothymoquinone (DBMIB) which causes a reduction of the plastoquinone pool, enhances the amount of damage (Kyle *et al.*, 1984a). Isolated pea thylakoids would most closely reflect a condition similar to that of DBMIB addition to whole cells. When electron flow was allowed to occur *in vitro* by the addition of ferricyanide, the extent of photodamage was reduced (Table I). Indeed, Kyle (1984) has recently shown that many environmental factors or mutations affecting CO_2 fixation lead to conditions of hypersensitivity to light damage.

The loss of Q_B-dependent electron flow has been shown to precede the Q_B-independent electron flow during photo-inhibition in intact *Chlamydomonas* cells (Kyle *et al.*, 1984a,b). This is also the case for photo-inhibition of isolated pea thylakoids, although the rate of degradation is somewhat faster in the *in vitro* system. Nevertheless, we note that although the Q_B-protein is the primary site of light damage, the PS II reaction center also appears to be damaged and degraded thereafter in isolated thylakoids. This light-induced differential loss of PS II electron transport activity is an important consideration when carrying out routine photochemical assays with isolated thylakoids.

The 'signal' for the damaged Q_B-protein and the subsequent proteolysis with no apparent formation of membrane-bound proteolytic fragments represent two important areas for future investigation. Mattoo *et al.* (1981) have reported that light incubation of *Spirodella* increases trypsin sensitivity of the Q_B-protein of isolated thylakoids. Levine (1983) has described the degradation of glutamine synthase in *E. coli* as occurring in two steps: (i) the oxidation of a single histidine residue rendering the enzyme inactive, and (ii) proteolysis by a protease specific for the modified enzyme. This single oxidation, however, was not accompanied by any major changes in protein structure or antigenicity (Levine *et al.*, 1981). An alternative mechanism of 'flagging' damaged proteins for proteolytic degradation involves the covalent attachment of a small (mol. wt. 8500) peptide-ubiquitin (Hershko, 1983). Since the ubiquitin mechanism is both ATP requiring and not membrane associated, it seems unlikely that it is involved in the degradation of the Q_B-protein. Thus, the actual mechanisms of 'flagging' and protein removal remain, for the present, obscure. The use of the described *in vitro* thylakoid system, however, should facilitate the elucidation of these mechanisms.

Materials and methods

Preparation of [³⁵S]methionine-labeled thylakoids

Pea plants (*Pisum sativum*) were grown for 11–14 days (16 h light/8 h dark) at 20°C on vermiculite watered with nutrient solution. Cool white fluorescent lights provided illumination intensities of 300–400 µE/m²/s. Plants of ~4–5 cm in length were cut above the seed and immersed in a solution of [³⁵S]methionine (500 µCi/ml of 1 mCi/µmol) in distilled water (50 µl per plant) in a 1 ml conical tube (Microfuge). The plants were exposed to a gentle stream of air and illuminated through a heat filter with a tungsten-halogen lamp at an intensity of 1000 µE/m²/s at the leaf surfaces. When the total volume of the [³⁵S]methionine solution was taken up (1.5–2 h), an additional amount of distilled water was added, and incubation was continued for a total duration of 3–4 h. Labeled plants were then rinsed briefly in distilled water and homogenized in grinding buffer, consisting of 50 mM tricine (pH 7.8), 10 mM NaCl and 5 mM MgCl₂. The homogenate was filtered through 12 layers of cheesecloth; unbroken cells and cell debris were removed by centrifugation at 1000 g for 2 min and the thylakoids were pelleted from the supernatant by centrifugation at 5000 g for 5 min. The thylakoid pellet was removed and washed in the same buffer by centrifugation as above and either used fresh or frozen at –80°C until use. A specific activity of 6000–10 000 c.p.m./µg chlorophyll was routinely obtained with 30–50% of the membrane-bound radioactivity associated with the Q_B-protein.

Photo-inhibition of thylakoids and photochemical assays

Labeled, isolated thylakoids were resuspended in the grinding buffer (50 µg chlorophyll/ml) in a glass tube and illuminated (1000 µE/m²/s) in a temperature-controlled water bath (24°C) for various lengths of time before assay. For membrane protein analysis, the samples were centrifuged and the supernatant discarded. For membrane protein analysis, the samples were centrifuged and the supernatant discarded. In some experiments, where both supernatant and membranes were analyzed, the chlorophyll concentration was increased to 1 mg chlorophyll/ml and 300 µl incubation volumes were used for photo-inhibition. The reactions were stopped in these latter cases by the direct addition of the electrophoresis sample buffer.

The Q_B-dependent PS II assay (water to dichloroindophenol) was carried out according to Patterson *et al.* (1983). The Q_B-independent PS II assay (water to silicomolybdate) was carried out according to Barr *et al.* (1975) as modified by Kyle *et al.* (1984a).

Electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis in the presence of lithium dodecylsulfate (LDS-PAGE) and 4 M urea at 7°C was according to Kyle *et al.* (1984a). Autoradiography of the stained and dried gels was carried out using Kodak AR film and routine exposure times of 4–8 days. A 22-kD portion from the amino acid residue 52 to the carboxy terminus (Hirschberg and McIntosh, 1983) of the Q_B protein from *Amaranthus hybridus* was overproduced in *Escherichia coli*. The polypeptide was purified and used to raise antibodies in rabbits. Details of the preparation of the clones and antibodies will be described elsewhere (Hirschberg *et al.*, in preparation). The polyclonal antibodies recognized the Q_B-protein from other higher plants (maize) as well as from *Chlamydomonas* and cyanobacteria. Immunoblotting (Towbin *et al.*, 1976) was carried out prior to gel staining, using 0–22 µm pore size nitrocellulose paper. Blots were incubated with antibodies and then the nitrocellulose filter was incubated with protein A-alkaline phosphatase conjugate (Sigma), followed by reaction with 5-bromo-4-chloro-3-indolyle-phosphate in the presence of Nitroblue tetrazolium (Sigma) at pH 9.5. Binding sites of antibodies were seen on the filter as blue to violet bands.

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