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Rapid Report

Light-dependent degradation of the Photosystem II D1 protein is retarded by inhibitors of chloroplast transcription and translation: possible involvement of a chloroplast-encoded proteinase

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Abstract

The light-dependent degradation of the photochemical Reaction Centre II D1 protein was retarded in higher plant *Lemna gibba* when inhibitors of chloroplast transcription or translation were present. D1 degradation may correlate with turnover of a chloroplast-encoded proteinase, but this proteinase seems not to be the only enzyme involved. Based on current knowledge and the results presented here, a proposal is made as to the proteinase involved.

Keywords: Chloramphenicol; Clp proteinase; Photoinhibition; Photosynthesis; Pulse-chase; Rifampicin; (*L. gibba*)

The 32 kD D1 protein, encoded by the chloroplast gene *psbA*, forms a part of the photochemical reaction centre of Photosystem II (RC II) in the photosynthetic, oxygen-evolving organisms. A unique characteristic of D1 is that it turns over in a light-dependent manner and much faster than the other photosynthetic membrane proteins under high light intensities [1–5]. D1 turnover is related to photoinhibition, a phenomenon whereby photosynthetic activity decreases under light intensities greater than those saturating photosynthesis [1,2,4,5]. When D1 synthesis and replacement cannot compensate for its damage and degradation, photoinhibition occurs [2]. Mechanisms of D1 degradation have been proposed. High light can cause photoinactivation at the acceptor side or donor side of PS II which further induces damage or modification of the RC II-D1 subunit [4,5]. The D1 protein is therefore triggered for degradation, and then proteolysed enzymatically [4,5]. The primary cleavage site in D1 and the types of enzyme catalysing D1 degradation have been proposed [6–9], but the enzyme that proteolyses D1 has not been identified [5].

Under physiological conditions, degradation of D1 is accompanied by re-synthesis of D1 and its integration into the PS II complex [1,2,4,5]. In mature chloroplasts, the psbA transcripts seem to be abundant and stable. The light-dependent turnover of D1 seems therefore to occur at the post-transcriptional or translational level rather than at the transcriptional level [1,10].

A new observation was made in the work reported here. Degradation of D1 in high light was retarded by inhibitors of chloroplast-protein translation and chloroplast-RNA synthesis in higher plant *Lemna gibba*. D1 degradation may therefore correlate with turnover of a chloroplast-encoded proteinase.

Axenic cultures of *Lemna gibba* L. G3 were pre-cultured as described previously [11]. The plants were then grown for 6–9 days at a PFD of 250 μmol m^{-2} s^{-1} and temperature of 22°C. Plants at the four-frond stage were selected for each experiment.

Net photosynthetic rate was measured as CO_2-uptake using an infra-red gas analyser (URAS 2G) in a closed gas-exchange system described previously [12,13]. The light source was slide-projectors with built-in Calflex filters and an external water filter. Air was pumped through the plant chamber and regulated us-

Abbreviations: CAP, chloramphenicol; PFD, photon flux density; PS II, Photosystem II, Rif, rifampicin (3-(4-methylpiperazinyliminomethyl)rifamycin SV).

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ing a mass-flow flow meter. Temperature in the chamber during all experiments was regulated to 22°C using a circulating water bath [13,14].

Sixty plants were pulse-labelled for 35 min in 12 ml growth medium containing 240 μCi [^{35}S]methionine (> 1000 Ci mmol^{-1}, Amersham) under a PFD of 1000 μmol m^{-2} s^{-1}. The plants were then rinsed and chased [13,14] for 2.5 h at a PFD of 1500 μmol m^{-2} s^{-1} in the presence of the inhibitors with concentrations specified in the figure legends.

Thylakoid enriched membranes were prepared by homogenising the *Lemna* plants with cold Tricine-HCl buffer (50 mM Tricine, 10 mM NaCl, 5 mM $MgCl_2$ (pH 7.8)) [15]. The homogenate was filtered through 4 layers of fine nylon mesh and centrifuged at 12000 × *g* for 10 min. The thylakoid pellets were washed once by centrifugation to get rid of starch, resuspended with the Tricine-HCl buffer, and preserved at −80°C for further use.

Thylakoid membrane polypeptides were resolved by lithium dodecyl-sulfate polyacrylamide gel electrophoresis (LDS-PAGE) using 13% acrylamide with the addition of 4 M urea and a buffer system as described [16]. Samples containing equal amount of chlorophyll were applied for PAGE. Chlorophyll concentration was determined according to Arnon [17]. The gels were stained with Coomassie brilliant blue R and vacuum-dried. The Photosystem II D1 protein was detected by autoradiography [13,14] and immunoblotting [15]. The procedure for immunoblotting was to electro-transfer the unstained gels to nitrocellulose paper, incubate with monospecific anti-D1 antibody, and assay using ^{125}I-protein A as reported previously [18]. To quantify the D1 protein, the autoradiographs were scanned densitometrically using a computer-programmed scanner and analysed with the software program NIH-Image.

When the intact duckweed plants were exposed for 2.5 h to a PFD of 1500 μmol m^{-2} s^{-1}, which is about twice as high as that saturating photosynthetic activity, the rate of light-limited photosynthesis was decreased by 14% (Fig. 1A). In the presence of chloramphenicol (CAP), an inhibitor of chloroplast translation, photosynthetic activity was decreased drastically, with only 10% remaining after 2.5 h. This phenomenon, known as photoinhibition, was expected, since CAP effectively inhibited synthesis of the D1 protein, which is damaged and rapidly lost under high light levels [2]. In *Lemna* the loss of light-limited photosynthesis under such experimental conditions is due to the loss of electron transport from Q_A to Q_B [14].

Rifampicin (Rif) is an inhibitor blocking chloroplast DNA-dependent RNA polymerase [19–21]. Addition of Rif did not increase photoinhibition under high illumination (Fig. 1A). Doubling or tripling the concentration of Rif did not significantly change the results

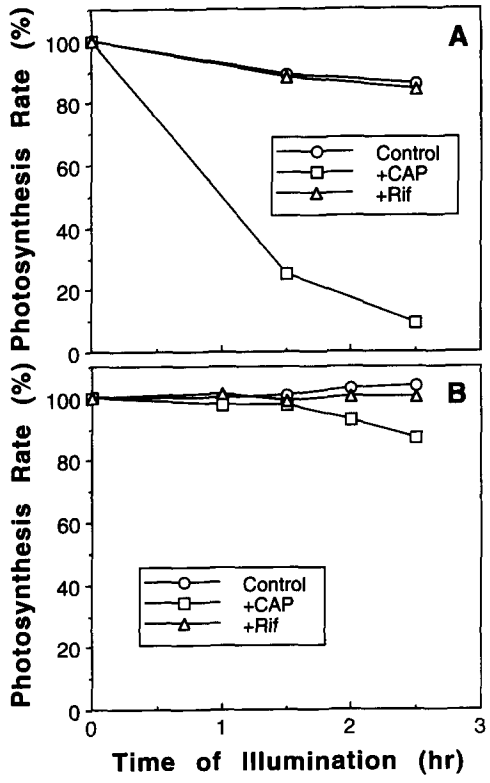

Fig. 1. Effect of CAP and Rif on photosynthesis and photoinhibition. Intact plants were illuminated under a PFD of 1500 μmol m^{-2} s^{-1} (A) or 250 μmol m^{-2} s^{-1} (B) in the absence of inhibitors (Control) or in the presence of 500 μg ml^{-1} CAP or 20 μg ml^{-1} Rif. Photosynthesis rate was measured as CO_2 uptake under PFD of 250 μmol m^{-2} s^{-1} (the same as growth PFD). Photosynthesis rate of 100% was 9.5 ± 0.4 μmol CO_2 m^{-2} s^{-1}.

(data not shown). When the plants were incubated with each of the inhibitors under growth light intensity, 250 μmol m^{-2} s^{-1}, CAP caused only a slight decrease in photosynthesis after 2 h, and Rif had no effect (Fig. 1B). Therefore, the inhibitory effect of CAP on photosynthesis (Fig. 1A) was light-dependent and closely related to protein turnover during photoinhibition.

Intact plants were pulse-labelled with [^{35}S]methionine and then the labelled proteins were chased under a PFD of 1500 μmol m^{-2} s^{-1}. The 32 kDa D1 protein was strongly labelled during the pulse and decreased (degradation) rapidly during the chase period, whereas the amount of the other thylakoid proteins remained stable (Fig. 2A). D1 degradation was retarded in the presence of CAP (Fig. 2). The $t_{1/2}$ for D1 degradation increased from 1.7 h in the control to 2.8 h in the presence of CAP (Fig. 2B). These results indicate that light-dependent degradation of D1 protein may require or be accompanied by chloroplast-protein synthesis. The newly synthesised protein(s) could be either D1 protein itself, or other proteins responsible for D1 degradation, e.g., proteinases. The former seems unlikely, because rifampicin retarded D1 degradation as

well as CAP did (Fig. 2). Unlike CAP, Rif did not enhance the sensitivity to photoinhibition (Fig. 1), and did not inhibit light-dependent D1 synthesis (see below, Fig. 3). A more likely explanation is that the inhibitors retard D1 degradation by inhibiting the synthesis of a protein different from D1.

To further clarify the effects of rifampicin as well as of chloramphenicol on the light-dependent D1 degradation, the total amount of D1 protein was measured by immunoblotting when the plants were exposed to high PFD (Fig. 3). In spite of re-synthesis of D1 during exposure to the photoinhibitory light, the net amount of D1 was decreased by 20% in the control after 2.5 h. D1 synthesis was blocked in the presence of CAP, and only about 35% of the D1 protein was left after 2.5 h (Fig. 3B). Although CAP retarded D1 degradation (Fig. 2), it could not completely prevent D1 from degradation (Fig. 2 and 3). In the presence of Rif, there was no apparent net loss of D1 (Fig. 3), although the pulse-chase experiment (Fig. 2) shows that D1 is degraded under these conditions. This clearly demonstrates that Rif, an inhibitor of chloroplast transcription, does not inhibit the light-dependent D1 synthesis. The net amount of D1 was more stable in the presence of Rif than that in the control (Fig. 3), showing that Rif retarded D1 degradation.

The results presented above show that D1 degradation is retarded by rifampicin as well as by chloramphenicol. This indicates that light-dependent degradation of the D1 protein correlates with turnover of a protein encoded by the chloroplast genome and responsible for D1 degradation. This inhibitor-sensitive protein could be D1 itself, a regulatory protein, or a proteinase. If it is D1, then the D1 degradation should not be retarded by Rif, because the mRNA transcripts of D1 seem to be abundant and stable in mature chloroplasts [1,10]. Figs. 2 and 3 show that this is not the case. The inhibitor-sensitive protein described above is therefore unlikely to be the D1 protein. Alternatively, the protein involved might be a regulatory factor that controls the proteolysis of D1, although there is at present no experimental work to support this possibility. The number of functional proteins encoded by chloroplast genome is limited, and so far no such regulatory factor protein has been identified. The results and the current knowledge seem to favour the possibility that a

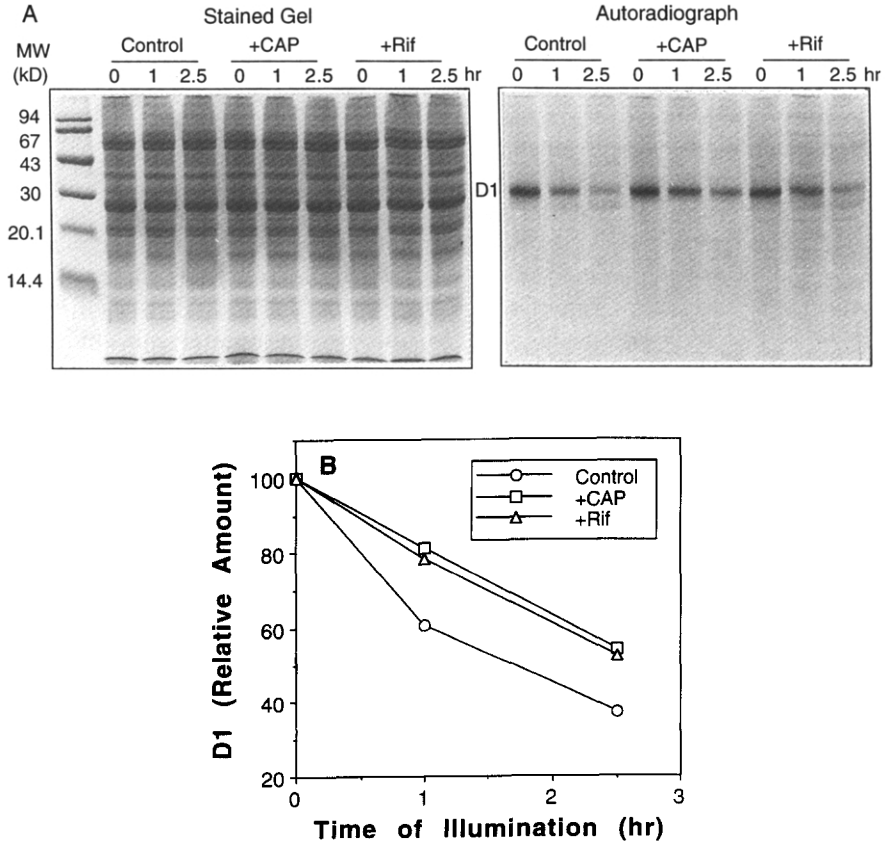

Fig. 2. Effect of CAP and Rif on light-dependent D1 degradation. Plants were pulse-labelled with [^{35}S]methionine for 35 min under PFD 1000 μmol m^{-2} s^{-1}, and chased under PFD 1500 μmol m^{-2} s^{-1} for 2.5 h without (Control) or with addition of CAP or Rif in the same concentrations as in Fig. 1. Samples were taken at the time indicated. (A) Stained gel and autoradiograph; (B) densitometrically quantified D1 amount from panel (A) (mean of three replicates), showing kinetics of D1 degradation.

chloroplast-encoded proteinase is involved during the light-dependent D1 degradation. One more argument favouring this proposal is that a chloroplast-encoded proteinase has been identified [22–24] (see below).

To date, the proteinase catalysing D1 degradation has not been identified [5]. It has been reported previously that D1 degradation is catalysed by a serine-type proteinase [9] or by a thiol-endoproteinase [8]. The proteinase was previously proposed to be the PS II chlorophyll *a* protein CP43 [25], but this seems to have come into doubt lately [5]. Another suggestion is that D1 degradation is auto-proteolytic [7]. Experiments with intact green algae have shown that the proteolysis of D1 requires re-oxidation of plastoquinol in the PQ pool [15]. Thus, different mechanisms may operate in vivo and in vitro. It has lately been suggested that there may be more than one proteinase activity required for the D1 degradation [5]. One of the most important chloroplast proteinases has been identified as the protein complex Clp A/P, an ATP-dependent internal serine-type proteinase [22,24]. The Clp A/P catalyses degradation of damaged or abnormal proteins [22,24]. The genes for the Clp P polypeptide were identified in open reading frames in the chloroplast genome of several higher plants, having a high degree of homology with *Escherichia coli* Clp ATP-dependent proteinase [22–24]. The Clp-like proteinases are highly conserved in plant chloroplasts and possibly in all organisms [22,24]. It is possible that the Clp A/P complex is involved in catalysing D1 degradation, and that the proteinase turns over during the process of catalysis. This suggestion is supported by reports indicating that D1 degradation is catalysed by a serine proteinase [5,9]. To clarify the role of Clp A/P in D1 degradation, however, requires more experiments. One could, for example, mutate the gene for Clp P and examine the effect on D1 degradation.

The present results indicate that the chloroplast-encoded proteinase is not the only enzyme involved in D1 degradation. In the presence of CAP or Rif, D1 degradation was retarded, but not fully stopped (Figs. 2 and 3). The net amount of D1 was lost by 60% after 2.5 h, in spite of the presence of CAP (Fig. 3). Also, the degradation of D1 protein over time was almost linear in the presence of Rif and CAP (Fig. 2). If the proteinase were the only protein involved in D1 degrada-

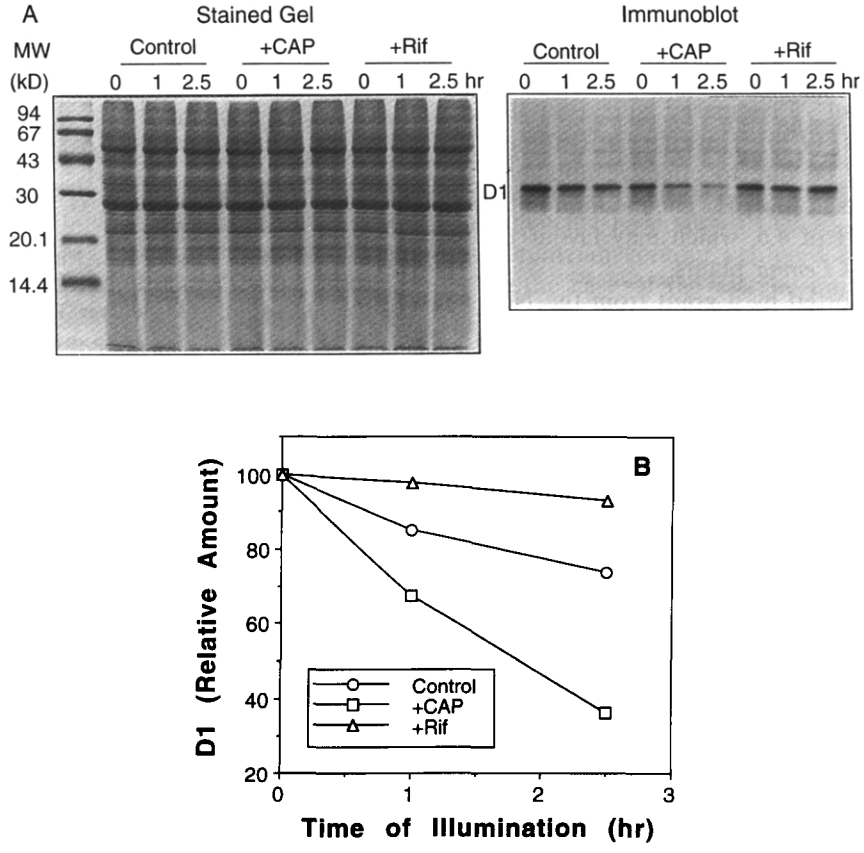

Fig. 3. Effect of CAP and Rif on total amount of D1 during photoinhibitory illumination. The plants were illuminated under a PFD of 1500 μmol m^{-2} s^{-1} with or without addition of CAP and Rif. D1 was quantified at the time indicated. (A) Stained gel and immunoblot; (B) D1 amount densitometrically quantified from (A) (mean of three replicates) showing the kinetics of D1 change under photoinhibitory PFD.

tion, then the degradation should be faster at 1 h than at 2.5 h, due to a relative abundance of the enzyme at the beginning. These results suggest that other enzyme(s) besides Clp P may be catalysing D1 degradation under the experimental conditions. Indeed, there have been reports showing that D1 degradation can occur without further addition of ATP in well-washed thylakoid membranes [5] and even in isolated RCIIs [5,7], whereas the Clp A/P is located in the stromal phase of chloroplast. It is possible that the chloroplast-encoded proteinase, if it is Clp P, functions in a secondary pathway in catalysing D1 degradation under physiological conditions.

Rifampicin inhibits RNA transcription by blocking the DNA-dependent RNA polymerase in bacteria [26] and chloroplasts [19–21]. The effect of Rif on chloroplasts was controversial, since a much higher concentration was often required in chloroplasts than in bacteria [26]. Previously it was not clear whether Rif inhibited the transcription of mRNA in chloroplasts, although there have been reports of Rif inhibiting synthesis of chloroplast total RNA and rRNA [19,20]. Recently, it has been shown that the bacteria-like B-enzyme of RNA polymerase in chloroplasts was severely inhibited by Rif in extremely low concentration [21]. This finding has indicated the possibility that Rif does inhibit the chloroplast mRNA transcription. The results in Figs. 2 and 3 show that Rif had an inhibitory effect on D1 degradation, at a relatively low concentration (20 μg ml^{-1}) compared with a number of previous experiments [19,20]. The effect of Rif in the low concentration may be due to effective uptake of Rif into the chloroplasts at low pH. The medium in the present work had a pH of 4.6, which may favour the uptake of rifampicin by *Lemna* plants.

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