Degradation of the 32-kilodalton thylakoid-membrane polypeptide of *Chlamydomonas reinhardi* Y-1

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**Abstract.** Isolated thylakoid membranes of *Chlamydomonas reinhardi* Y-1 with the 32-kDa polypeptide either radioactively labelled or unlabelled were incubated in vitro under various conditions in order to gain information about the degradation of the 32-kDa polypeptide. The degradation was higher at pH 6 compared with pH 7 and pH 8 and exhibited a temperature maximum between 20° and 25° C (pH 6, pH 8). A light-dependent part of the total degradation was linearly dependent on white light of energy fluence rate between 1 and 20 mW·cm⁻² at 25° C and leveled out at higher fluence rates. The degradation in light was only slightly stimulated by ATP but was reduced by 3-(3',4'-dichlorophenyl)-1,1-dimethylurea. Adenosine-5'-diphosphate and heparin (2.7 mM and 200 µg per 100 µl, respectively) known to inhibit kinases, caused a 50% decrease in degradation indicating that a phosphorylation step is involved in degrading the 32-kDa polypeptide. Out of various inhibitors specific for different types of proteases, only those for thiol- and endoproteases showed intense effects. These results point to a proteolytic degradation of the 32-kDa polypeptide by a thylakoid-membrane-bound thiol-endoprotease. Its activity yields soluble breakdown products with relative molecular masses (M₉) of 23, 16.5, 11.3 and 10.7 kDa, and these are accumulated in the in-vitro system. Partial proteolytic digestion of thylakoids with *Staphylococcus aureus* V8 protease results in at least two labelled breakdown products (M₉ 23, and 16.5 kDa). It is assumed that cleaving at identical amino-acid residues of the 32-kDa polypeptide by the thylakoid-membrane-bound thiol-

endoprotease and the V8 protease results in these two breakdown products. They are derived from subsequent cleavage at amino-acid residues 60-242 and 60-189 according to the deduced protein sequence (Erickson et al. 1984, EMBO J. 3, 2753–2762).

**Key words:** *Chlamydomonas* – Herbicide binding protein – Polypeptide (thylakoid 32-kDa) – Thylakoid 32-kDa polypeptide.

**Introduction**

Recently, much attention has been focussed on the 32-kDa polypeptide of the thylakoid membranes from higher plants and algae. This polypeptide is a minor component in the thylakoids, its structural gene has been mapped to the chloroplast DNA in the large single-copy region, immediately adjacent to one of the inverted repeat regions (Bredbrook et al. 1978), and it has been cloned (Bogorad et al. 1980). It is synthesized as a precursor at ribosomal binding sites on stroma lamellae and, in a post-translational step, processed to its mature form still membrane-bound (Marder et al. 1982; Herrin and Michaels 1985).

The protein or its precursor species is rapidly labelled (Edelman and Reisfeld 1978; Wettern et al. 1983) and has a light-stimulated fast turnover (Reisfeld et al. 1978; Owens et al. 1982). In *Chlamydomonas reinhardi* Y-1 the 32-kDa polypeptide is phosphorylated in light and darkness but the degree of phosphorylation can be enhanced by light and this seems to be important for the structural integration and function within active photosystem (PS) II units (Owens and Ohad 1983). In these units the 32-kDa polypeptide functions as an apoprotein of “B” (Arntzen et al. 1984), and
appears to be the binding site for herbicides such as 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) and atrazin (Pfister et al. 1981; Oettmeier et al. 1983).

In our studies on the turnover of the 32-kDa polypeptide in *C. reinhardi* Y-1, we found the degradation in vivo to be strictly light-stimulated and inhibited by DCMU, indicating that its proteolysis needs an active electron flow via PSII (Owens et al. 1982; Wettern et al. 1983). In addition, the degradation does not immediately follow the end of a pulse (Wettern 1984). A lag phase of about 4 h could be observed before a decrease of radioactivity incorporated into the 32-kDa polypeptide occurred (Wettern and Ohad 1984).

To obtain further information about the conditions for degradation of the 32-kDa polypeptide, isolated thylakoids of *C. reinhardi* Y-1 were studied in a cell-free system.

### Material and methods

*Organism, growth condition and labeling with $^{35}$S-sulphate.* Cells of *Chlamydomonas reinhardi* Y-1 were grown on a mineral medium containing acetate (Ohad et al. 1967) and harvested at the end of logarithmic growth. To label thylakoid membranes the collected cells were washed once and resuspended in fresh growth medium with 0.2 μM sulphate at a concentration of 2·10^7 cells·ml⁻¹. The labelling was done with a specific radioactivity of 7.54·10^7 Bq Na$_2^{35}$SO$_4$ (Amersham-Buchler, Braunschweig, FRG) per 1.0 μM sulphate for 30-60 min in light at 25°C as described by Wettern et al. (1983).

*Isolation of thylakoid membranes and fractionation of thylakoid-membrane protein.* To obtain thylakoid membranes, cells were washed with non-radioactive growth medium, resuspended in an appropriate medium and broken with glass beads (0.25–0.30 mm) within 1 min in a “Vibrogen Cell Mill” (Bühler, Tübingen, FRG). The homogenate was decanted from the glass beads and a pellet was obtained by centrifugation of this homogenate for 30 min at 80000 g in a SW-27 rotor (Beckman, München, FRG). Thylakoid membranes were isolated by gradient-centrifugation of this pellet in a procedure described by Owens and Ohad (1982), fractionated by lithium-dodecylsulphate-polyacrylamide gel electrophoresis (LDS-PAGE) according to Chua (1980) and fluorographed after staining for proteins with Serva-blue according to Wettern et al. (1983).

*In-vitro incubation of thylakoids.* For measurement of protein degradation in a cell-free system from thylakoid membranes, either $^{35}$S-labeled (120 μg protein per 100 μl) or unlabelled membranes (various concentrations) were incubated – unless otherwise stated – in 30 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), pH 6; 5 mM ATP; 10 mM MgCl$_2$; 25°C; white light; according to Wettern (1984). After centrifugation of the sample at 30000 g for 10 min the radioactivity in the supernatant was determined with a liquid scintillation counter (LS 9000; Beckman).

Azocoll (Sigma, München, FRG) digesting activity was determined by measuring the release of the red dye from the substrate. The substrate (various amounts per 1 ml) was incubated with thylakoid membranes in the buffer described above and after centrifugation at 30000 g for 10 min the supernatant was measured at 520 nm against a blank containing boiled sample.

In testing the effect of different protease inhibitors on proteolytic degradation, various inhibitors were assayed using the system described above.

Soluble proteins were precipitated with trichloroacetic acid (TCA) at a final concentration of 5%. The method of Cleveland et al. (1977) was used for limited proteolysis of the 32-kDa polypeptide with Staphylococcus aureus V8 protease (Sigma, Taufkirchen, FRG).

### Results

The turnover in vivo of the 32-kDa polypeptide is known to be strongly light-stimulated. Therefore, we tested the in-vitro effect of various energy fluence rates of white light on the release of radioactivity from prelabelled thylakoids. The labelling pattern of membranes used is given in Fig. 1. The

![Fig. 1. Pattern of $^{35}$S-labelling of thylakoid-membrane protein from *C. reinhardi* Y-1 used in the cell-free system. Cells of *C. reinhardi* Y-1 were labelled and thylakoid membrane proteins were fractionated with 15% polyacrylamide gels as described in Material and methods. For separation, 120 μg protein was loaded on one slot. The staining for protein with Serva-blue is given in lane 1, the fluorogram in lane 2.](attachment:image)