Intrinsic membrane proteins of the thylakoids of *Chlamydomonas reinhardii*

HANS PETER MICHEL, ERASMUS SCHNEIDER, MATTHIAS TELLENBACH and ARMINIO BOSCHETTI*

Institut für Biochemie der Universität Bern, Freiestrasse 3, 3012 Bern, Switzerland

(*Received 8 April 1981*)

Key words: apoproteins of the LHCP, one-dimensional fingerprints, pronase treatment of thylakoids, *Chlamydomonas* proteins

Abstract. Upon proteolytic treatment of thylakoid membranes, extrinsic proteins are selectively degraded. The remaining resistant proteins have been analyzed by SDS-PAGE. In the thylakoids of *Chlamydomonas reinhardii*, six polypeptides or protein fragments of 20 kD or higher are resistant to proteolysis. These intrinsic proteins have been identified as: the apoproteins of the chlorophyll-protein complexes CP I and LHCP; a polypeptide whose presence is related to the chlorophyll *b* content of the cells; and a portion of a chloroplast-made 34 kD polypeptide. Furthermore, after proteolytic treatment of the membranes, the LHCP complex can be resolved into two subcomplexes, apparently differing in their polypeptide composition.

Zusammenfassung. Durch proteolytische Behandlung von Thylakoidmembranen werden die extrinsischen Proteinanteile selektiv abgebaut. Die resistenten, in der Membran verbleibenden Polypeptide können mit SDS-PAGE analysiert werden. In Thylakoiden von *Chlamydomonas reinhardii* finden sich sechs resistente Polypeptide mit Molekulargewichten über 20'000. Durch quantitative Bestimmung während der Proteolyse und durch limitierte Fragmentierung nach Cleveland wurden die resistenten Polypeptide identifiziert als: (a) Apoproteine von CP 1, (b) Apoproteine des LHCP-Komplexes, (c) Teil des chloroplastidial synthetisierten 34 kD-Proteins und (d) Polypeptid, welches möglicherweise mit dem Chlorophyll *b*-Gehalt in Verbindung steht. Im weiteren führte die proteolytische Behandlung der Thylakoide zu einer Auftrennung des LHCP-Komplexes in zwei Subkomplexe mit unterschiedlicher Proteinzusammensetzung.

Introduction

The treatment of membranes with proteolytic enzymes has often been used as a tool for identifying those proteins that are intrinsic to the membrane. Under controlled conditions, the proteases are believed to attack the polypeptides exposed on the surface, while those polypeptides that are embedded in

*To whom correspondence should be addressed.

Abbreviations: CP 1 = pigment-protein complex with slower mobility in SDS electrophoresis, corresponding to the P700 chlorophyll *a* protein complex; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; EDTA = ethylene-diaminotetraacetic acid; kD = kilo Dalton; LHCP = pigment-protein complex with intermediate mobility in SDS electrophoresis, corresponding to the light-harvesting chlorophyll *a*/*b* protein complex; SDS = sodiumdodecyl sulfate; SDS-PAGE = electrophoresis in SDS-containing polyacrylamide gels; Tris = tris (hydroxymethyl) aminomethane.

Photosynthesis Research 2: 203–212 (1981) 0166–8595/81/0023–0215 $01.50.

the membrane remain unaltered. The resulting SDS-polyacrylamide gel electropherograms obtained from protease-treated membranes present a much simpler pattern with fewer polypeptides than those from untreated membranes. The remaining polypeptides that are resistant to relatively high concentrations of protease are presumed to represent the intrinsic membrane proteins. Such studies have been applied to the thylakoids of higher plants [5, 15, 16] and of green algae [14, 7]. In both cases protease resistant membrane proteins and fragments have been found mainly in chlorophyll-protein complexes. The definitive identification of the resistant protein fragments with the native membrane proteins has only been proved in a few instances.

In our studies with the thylakoids of *Chlamydomonas reinhardii*, we found that while many polypeptides were fully susceptible to pronase digestion, a few were quite resistant and apparently protected from degradation. In this report, we describe the characterization of the fully resistant polypeptides as well as of the relatively large fragments (over 20 kD) remaining after pronase digestion. We show that these fragments arise out of specific native polypeptides and have confirmed their identities by quantitation of the products of progressive digestion and by the technique of one-dimensional fingerprinting [8]. Furthermore, pronase treatment enables us to show that the LHCP complex consists of at least two subcomplexes; and we have also identified their apoproteins.

Materials and methods

The conditions for the growth of *Chlamydomonas reinhardii* $Arg^{-}mt + sr_3$ as well as the isolation of the thylakoids were as described previously [4]. In certain experiments, it was desirable to use cells with radioactively labelled chloroplast-made proteins. For this purpose, 3 liters of culture at a density of 6×10^6 cells/ml were incubated with 0.5 mCi $NaH^{14}CO_3$ (54.5 mCi/m*M*, Amersham) and 2 μg/ml cycloheximide for 3 hours prior to harvesting.

Treatment with protease. After isolation, the thylakoids were washed with EDTA [2] and then resuspended in 0.1 *M* Tris/HCl, pH 7.6 to a concentration of 1 mg chlorophyll/ml. The membranes were treated with pronase or trypsin (both from Sigma) at 35°C. The reaction was stopped by 3 successive washings of the membranes with the same buffer which contained, in addition, 50 m*M* 2-mercaptoethanol, 5 m*M* EDTA and 0.01% phenylmethylsulfonylfluoride (Sigma).

Electrophoresis of the proteins. For the analysis of the polypeptides, the samples were electrophoresed on SDS-polyacrylamide slab gels, using the buffer system of Laemmli [12]. We obtained a rather good resolution when the acrylamide and bisacrylamide solutions were freshly prepared. The gels

contained a linear gradient of 10 to 25% acrylamide. Preparative electrophoresis was carried out on gel slabs (200 x 100 x 3.5 mm) containing 10% acrylamide.

Membrane samples were solubilized either completely (2% SDS; 1% 2-mercaptoethanol; 4 min at 90°C) or partially to obtain the chlorophyll-protein complexes (1% SDS; 30 min at 0°C). The chlorophyll concentration was 1 mg/ml. Electrophoresis of fully solubilized samples was carried out at room temperature while the partially solubilized samples were run at 4°C. After electrophoresis, the gels were either stained with Coomassie Brilliant Blue, or exposed on Kodak X-ray films (18 x 24 cm) in the case of radioactively-labelled samples.

One-dimensional fingerprinting. This method has been previously described [8]. Limited proteolytic digestion was obtained with *Staphylococcus aureus* protease V8 (Miles) or papain (Sigma). The samples consisted on gel pieces (0.5 to 1 cm long) cut out of preparative gels which had been stained with Coomassie Brilliant Blue. A fresh gel containing 15% polyacrylamide was prepared. The sample wells of the stacking gel were first filled with half of the protease solution to be used. After 12 minutes of pre-electrophoresis at room temperature with a current of 9 mA/cm^2, the gel pieces were introduced into the wells and overlaid with the remaining half of the protease solution. Partial digestion of the samples was allowed to proceed at room temperature by interrupting the electrophoresis twice for 15 min each time, when the Bromphenol Blue marker had reached one third and then two thirds of the length of the stacking gel.

Results

EDTA-washed thylakoids of *Chlamydomonas reinhardii* yield a typically complex pattern of polypeptides upon SDS-PAGE (Figure 1). Mild treatment of these membranes with pronase (less than 1 μg/mg membrane protein) results in the fairly rapid disappearance of certain polypeptides, notably those at 29, 32 and 70 kD. However, it can be seen that certain polypeptide fragments persist, even after prolonged exposure (30 to 60 min) to the pronase.

Indeed, under more drastic conditions, using higher concentrations of proteolytic enzyme (over 10 μg/mg membrane protein), we find that in addition to the smaller fragments, there are about 6 polypeptides or fragments above 20 kD which are resistant to digestion (Figure 2). Very similar protein patterns are obtained using pronase or trypsin, indicating that the selectivity of degradation is not due to the specificities of the enzymes. The polypeptides of 25 and 20 kD are completely resistant to protease digestion and seem to be intrinsic proteins (Figure 2A and 2B). Two other bands at 29 and 24 kD disappear, with the concomitant appearance of two new bands at 27.5 and 23 kD, respectively. These changes can be seen more clearly when the samples

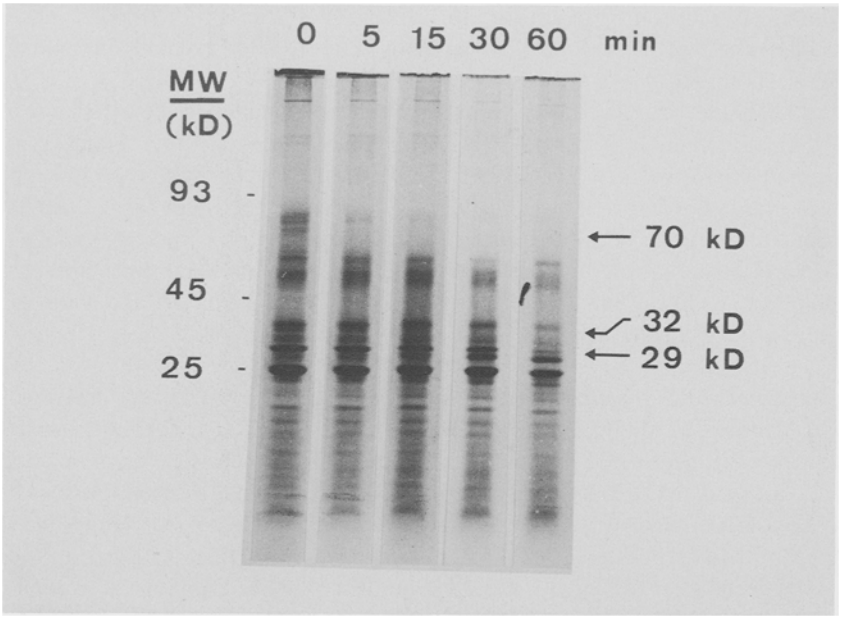

Figure 1. Time-dependent digestion of EDTA-washed thylakoids with pronase (0.7 μg per mg membrane protein). SDS-electrophoresis on a 10–25% polyacrylamide gradient gel; stained with Coomassie Brilliant Blue

are run on 10% polyacrylamide gels, where the resolution in this molecular weight range is better (Figure 2B).

It was also of interest to us to determine the effect of pronase on those membrane proteins which are synthesized in the chloroplast. We therefore prepared EDTA-washed thylakoids from cells which had been given $NaH^{14}CO_3$ in the presence of cycloheximide. Upon electrophoresis and autoradiography, a greatly simplified pattern is obtained (Figure 2C). The major bands correspond to the apoprotein of the CP I at about 75 kD and the 34 kD polypeptide, both of which had been shown to be synthesized in the chloroplasts [6]. The 34 kD polypeptide is normally not seen with Coomassie staining. One of the two bands intermediate between the CP I apoprotein and the 34 kD polypeptide may correspond to the unresolved α and β subunits of the chloroplast coupling factor CF_1 which were not fully extracted with EDTA. When the membranes are treated with pronase, the radioactive band corresponding to the apoprotein of CP I remains, whereas the 34 kD polypeptide mostly disappears. A strong band of radioactivity now appears at about 21 kD.

We then set out to establish the identities of the polypeptide fragments obtained after pronase digestion with the native membrane proteins, using two methods. In the first, the relative intensities of the stained bands before and after progressive digestion with pronase were compared. Thus, from a semi-quantitative evaluation of the bands in Figure 1, we infer that the

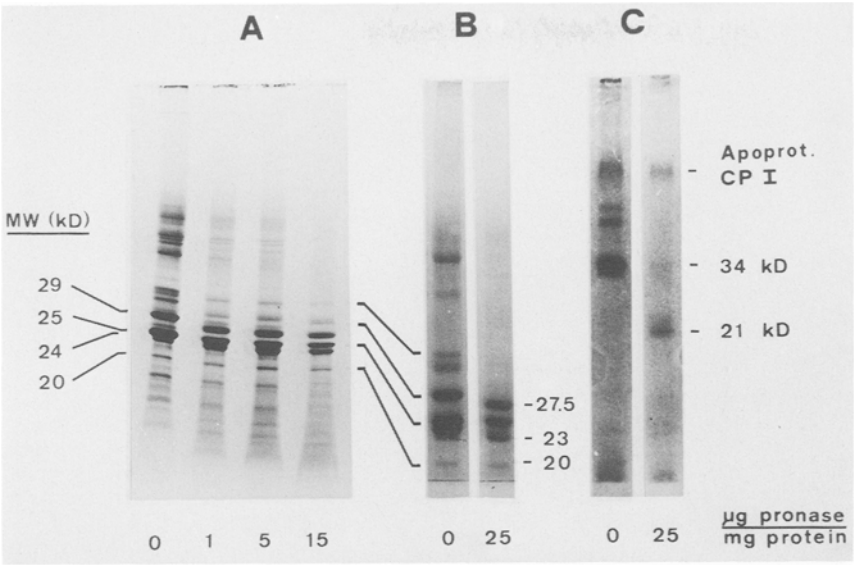

Figure 2. Digestion of EDTA-washed thylakoids with different amounts of pronase (each 15 min). (A) Separation on a 10–25% polyacrylamide gradient gel, stained with Coomassie Brilliant Blue. (B) Separation on a 10% polyacrylamide gel, stained with Coomassie Brilliant Blue. (C) Autoradiograph of a separation of thylakoid membranes of cells whose chloroplastidially synthesized proteins have been radioactively labelled; 10–25% polyacrylamide gradient gel

27.5 kD fragment arises out of the 29 kD polypeptide. An analogous evaluation of the bands of the autoradiograms in Figure 2C indicates that the 21 kD fragment comes from the rapidly labelled 34 kD polypeptide. At the same time, a comparison of the radioactivities of the CP I apoprotein bands shows that this protein is rather resistant to pronase digestion.

A more unambiguous method for confirming the identities of the polypeptides is that of limited proteolysis [8] of proteins during SDS-PAGE. After electrophoresis, the resulting pattern of peptide fragments is quite distinctive of the particular protein substrate. Such patterns of peptide fragments from specific polypeptides before and after pronase treatment of the membranes are illustrated in Figure 3. The 29 kD polypeptide and the 27.5 kD polypeptide, which appears after pronase digestion, show several peptide fragments in common (Figure 3A). Two of the peptide fragments from the original 29 kD polypeptide are not present in the 27.5 kD cleavage product; instead, two peptide fragments with correspondingly higher mobilities appear (indicated by slanted lines in the figure). The peptide fragments with altered mobilities may belong, in part, to the segment of the polypeptide that is attacked by pronase. These results strongly indicate that the 27.5 kD polypeptide comes from the 29 kD polypeptide. The other polypeptide which is susceptible to pronase is that at 24 kD. When the peptide fragments of this band are compared with those of the 23 kD polypeptide which appears

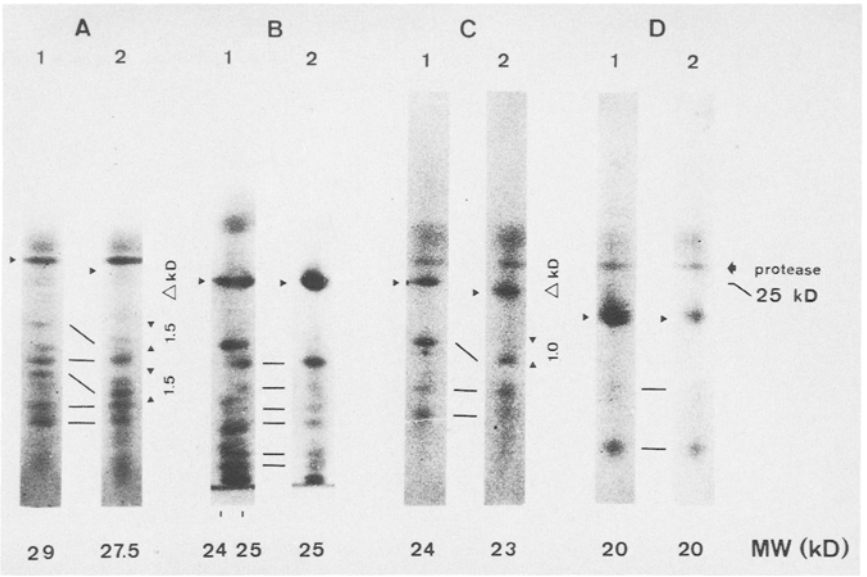

Figure 3. Partial digestion with *S. aureus* protease V8 of individual proteins isolated from (1) untreated membranes and (2) pronase treated membranes. ▶: Position of the undigested protein. The fingerprints are arranged in pairs (A-D) to show the relationships. (A, C, D): 0.75 μg *S. aureus* protease; (B) 0.25 μg *S. aureus* protease

as a consequence of pronase treatment of the membranes, we also find peptide fragments common to both (Figure 3C), again indicating identity of these polypeptides.

As mentioned earlier, two polypeptides, one at 25 and the other at 20 kD appeared to be fully unaffected by pronase treatment of the membranes. Analysis of the bands excised from the electropherograms of untreated and treated membranes shows that in each case the peptide fragments are identical (Figure 3B and 3D). In most instances, it is very difficult to separate the 25 from the 24 kD polypeptides upon electrophoresis of untreated membranes. Therefore, the substrate shown in Figure 3B is a mixture of the two. However, the peptide fragments produced from each polypeptide can be partially resolved by placing the gel piece in the sample compartment of the second gel in a manner such that the 25 kD portion of the band is oriented towards the right, as shown in Figure 3B. The polypeptides of 29, 25 and 24 kD thus behave as intrinsic proteins, since they are fully or in large part protected from pronase digestion. These polypeptides have been found to belong to the LHCP [3].

It was therefore of interest to determine the effect on the LHCP complex of pronase treatment of membranes. Upon electrophoresis of membranes on SDS-polyacrylamide gels, under partially dissociating conditions, as described in 'methods', two green zones are obtained in addition to the free chlorophyll, corresponding to CP I and LHCP. Within the region of the LHCP, we find

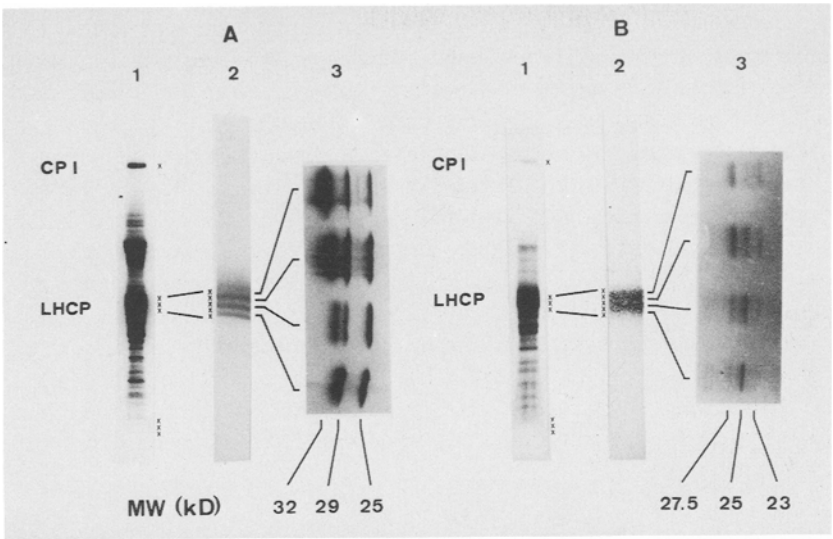

Figure 4. Re-electrophoresis of green LHCP without (A) and after (B) treatment with 1 μg pronase per mg membrane protein. (1) Gel stained with Coomassie Brilliant Blue. (2) Sections of an unstained gel showing the green LHCP. (3) Re-electrophoresis of individual green bands cut out from (2) and stained with Coomassie Brilliant Blue. All gels contain 10–25% polyacrylamide. x: green bands

three to five rather sharp green bands, quite close to each other (Figure 4A-2). By cutting out each individual band and subsequent re-electrophoresis, this time under dissociating conditions, the polypeptide composition of the different bands in the complex may be compared. After staining, the corresponding patterns of polypeptides in Figure 4A-3 are obtained. It can be seen that two bands in particular (29 and 25 kD) appear in all of the electropherograms. The other stainable polypeptides in the region of 30 to 32 kD are probably due to incompletely dissociated complexes or are polypeptides which comigrate with the green bands. Further faintly-staining polypeptides also appear below 20 kD.

When pronase-treated thylakoid membranes are similarly analyzed we find the following: the LHCP complex has a similar mobility to that of the LHCP of untreated membranes. However, instead of the sharp bands, the LHCP from pronase-treated membranes now contains only two rather diffuse green bands (Figure 4B-2). When these bands are cut out and sliced into four different samples and re-electrophoresed we find three major staining polypeptides at 27.5, 25 and 23 kD (Figures 4B-3). It should be recalled that the 27.5 and the 23 kD fragments derive from the 29 and 24 kD polypeptides, respectively, of untreated membranes. From the intensities of the stained bands there seems to be an enrichment of the 25 kD polypeptide in the lower, and of the 27.5 and 23 kD polypeptides in the higher molecular weight regions of the LHCP. All these results indicate that the LHCP complex consists of at least

three apoproteins of 29, 25 and 24 kD. Furthermore, it appears that the complex can be further resolved into subcomplexes; at least two of these subcomplexes are composed of the intrinsic apoproteins of the 29 and 24 kD on the one hand and the 25 kD polypeptide on the other.

The other intrinsic protein of the thylakoid membranes that is unaffected by pronase treatment is the 20 kD polypeptide. A clue to its function may be obtained from studies with two mutants which we have isolated from *Chlamydomonas reinhardii*. These mutants have reduced chlorophyll *b* content (chlorophyll *a*/*b*, 14.5 and 9.7) although they remain photoautotrophs. Upon SDS-PAGE of the thylakoid membranes isolated from these mutants we find some slight differences in the polypeptide composition as compared to those of the wild type. The most striking difference lies in the 20 kD band, which is much reduced in one mutant or even absent in the other. This polypeptide may thus be part of a chlorophyll *b* complex or even of the LHCP complex.

Discussion

In order to ascertain that the polypeptides which resist pronase treatment are indeed imbedded in the membrane and therefore intrinsic, one must rule out the possibility of selective proteolysis due to enzyme specificity or protection by carbohydrate residues on the proteins. The resistant polypeptides we have described in this paper are obtained with either pronase or trypsin. The thylakoid membranes of *Chlamydomonas reinhardii* have previously been treated with trypsin [3] and thermolysin [7], and the resulting polypeptide patterns were similar in both cases. Thus, enzyme specificity does not seem to be involved in selective proteolysis. On the other hand, the presence of carbohydrate residues on the polypeptides could protect them from proteolysis. However, we could not detect any significant amounts of such residues on the polypeptides in question, after carbohydrate staining with periodic acid-Schiffs reagent. Similar polypeptides from *Acetabularia mediterranea* have been shown to contain only about 1% of carbohydrate [1]. The protease resistant polypeptides therefore behave as intrinsic proteins.

We have shown that several thylakoid membrane proteins of *Chlamydomonas reinhardii* are fully or to a large extent resistant to protease digestion. Of these, we find that the 24, 25 and 29 kD polypeptides are apoproteins of the LHCP complex. These proteins show different fingerprints after limited proteolysis with *S. aureus* protease or papain, indicating that they are distinct from one another. The 24 kD protein may also be distinguished from the other two apoproteins in that the former becomes fully susceptible to pronase digestion when the cells are subjected to ultrasonication. It has also been reported [11] that the 29 and 26 kD proteins associated with the LHCP of *Chlamydomonas reinhardii* had different fingerprints after digestion with *S. aureus* protease, although they were chemically

related and structurally similar. From the behaviour of the polypeptides upon electrophoresis we can further resolve the LHCP complex into subcomplexes with different apoproteins. The LHCP from *Hordeum vulgare* [17] and *Vicia faba* [13] have previously been reported to consist of two chlorophyll-protein complexes differing in their polypeptide composition. The 29 kD protein may be involved in membrane stacking. In peas, [5] a 28 kD protein which is analogous to the 29 kD protein of *Chlamydomonas reinhardii* has been described. After mild proteolysis of the membrane, a 27 kD fragment appeared and a concomitant decrease in membrane stacking was observed.

It is interesting that the polypeptides which are intrinsic to the membranes are synthesized either outside or inside the chloroplast. The apoproteins of the LHCP complex have been reported to be synthesized in the cytoplasm [6]. On the other hand, the apoprotein of the CP I as well as a 34 kD polypeptide which has a high turnover are among the membrane proteins that have been shown to be synthesized in the chloroplast [6].

The highly labelled 34 kD polypeptide which gave rise to a 21 kD polypeptide fragment after pronase treatment of the membrane (Figure 2C) is probably analogous to the 34 kD membrane polypeptide containing a 19 kD protease resistant fragment that has been reported for maize [10]. This protein has recently been reported to contain a DCMU-binding site which is susceptible to trypsin digestion [9]. Indeed, it has previously been reported [14] that photosystem II activity was only slightly affected whereas the DCMU sensitivity was markedly reduced after pronase treatment of the thylakoid membranes of *Chlamydomonas reinhardii.* We also found that the photosystem II activity (reduction of 2,6-Dichlorophenolindophenol of ultrasonicated cells) is inhibited only to 40% by DCMU when previously treated with pronase, while in untreated preparations the inhibition is 100% with the same amount of DCMU.

Investigations into the identity and function of the thylakoid membrane proteins have been hampered by the complexity of these structures. From the present results, it is apparent that the controlled proteolytic treatment of thylakoids offers an invaluable tool for studying certain of their proteins. In addition to the identification of intrinsic membrane proteins and their fragments, these proteins become amenable to isolation in purer form. The further characterization of these proteins may eventually lead to an understanding of their function.

Acknowledgements

This work was supported in part by the Swiss National Science Foundation.

References

1. Apel K (1977) The light-harvesting chlorophyll *a/b*-protein complex of the green alga *Acetabularia Mediterranea.* Isolation and characterization of two subunits. Biochim Biophys Acta 462: 390–402.

2. Apel K, Miller KR, Bogorad L and Miller GJ (1976) Chloroplast membranes of the green alga *Acetabularia Mediterranea.* J Cell Biol 71: 876–893.
3. Bar-Nun S, Schantz R and Ohad I (1977) Appearance and composition of chlorophyll-protein complexes I and II during chloroplast membrane biogenesis in *Chlamydomonas reinhardi* y-1. Biochim Biophys Acta 459: 451–467.
4. Boschetti A, Sauton-Heiniger E, Schaffner JC and Eichenberger W (1978) A two-dimensional separation of proteins from chloroplast thylakoids and other membranes. Physiol Plant 44: 134–140.
5. Carter DP and Staehelin LA (1980) Proteolysis of chloroplast thylakoid membranes. I. Selective degradation of thylakoid pigment-protein complexes at the outer membrane surface. Arch Biochem Biophys 200: 364–373.
6. Chua NH and Gillham NW (1977) The sites of synthesis of the principal thylakoid membrane polypeptides in *Chlamydomonas reinhardi.* J Cell Biol 74: 441–452.
7. Chua NH and Blomberg F (1979) Immunochemical studies of thylakoid membrane polypeptides from Spinach and *Chlamydomonas reinhardtii.* J Biol Chem 254: 215–223.
8. Cleveland DW, Fischer SG, Kirschner MW and Laemmli UK (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 252: 1102–1106.
9. Edelman M, Mattoo AK and Hoffman-Falk H (in press) Regulation and Identification of translation products in the chloroplast. In: Fifth International Congress on Photosynthesis 1980 (Akounoglou et al eds).
10. Grebanier AE, Coen DM, Rich A and Bogorad L (1978) Membrane proteins synthesized but not processed by isolated maize chloroplasts. J Cell Biol 78: 734–746.
11. Hoober JK, Millington RH and D'Angelo LP (1980) Structural similarities between the major polypeptides of thylakoid membranes from *Chlamydomonas reinhardtii.* Arch Biochem Biophys 202: 221–234.
12. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
13. Machold O and Meister A (1979) Resolution of the light-harvesting chlorophyll a/b-protein of Vicia Faba chloroplasts into two different chlorophyll-protein complexes. Biochim Biophys Acta 546: 472–480.
14. Regitz G and Ohad I (1976) Trypsin-sensitive photosynthetic activities in chloroplast membranes from *Chlamydomonas reinhardi*, y-1. J Biol Chem 251: 247–252.
15. Steinback K, Burke JJ and Arntzen CJ (1979) Evidence for the role of surface-exposed segments of the light-harvesting complex in cation-mediated control of chloroplast structure and function. Arch Biochem Biophys 195: 546–557.
16. Süss KH, Schmidt O and Machold O (1976) The action of proteolytic enzymes on chloroplast thylakoid membranes. Biochim Biophys Acta 448: 103–113.
17. Süss KH and Brecht E (1980) Polypeptide composition and spectral properties of light-harvesting chlorophyll a/b-protein complexes from intact and trypsin-treated chloroplast thylakoid membranes. Biochim Biophys Acta 592: 369–374.