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THE ACTION OF PROTEOLYTIC ENZYMES ON CHLOROPLAST THYLAKOID MEMBRANES

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SUMMARY

Envelope- and stroma-free thylakoid membranes of *Vicia faba* chloroplasts were incubated with trypsin or pronase for several hours. The indigestible residue was analysed by polyacrylamide gel electrophoresis. Trypsinization resulted in a complete digestion of all proteins with the exception of the pigment-protein complexes as well as a polypeptide not yet characterized. Yet, as compared with untreated material, Complex II was found to have higher electrophoretic mobility. Electron-microscopic studies illustrate that the indigestible residue still has a preserved membrane structure. Disintegration of the thylakoid membranes by sodium dodecyl sulfate followed by trypsinization also resulted in the two complexes while all the other proteins were found to be digested. However, after removal of the lipids the protein moieties of the complexes proved to be easily digestible. From these results it is concluded that pigment-protein interaction may be an important factor in maintaining a conformation rather resistant to perturbants and proteases. In contrast to trypsin, pronase completely digested the polypeptides of the thylakoid membranes including the protein moieties of the pigment-protein complexes leaving an amorphous lipid mass. The results support the assumption that the complexes are necessary to maintain the membrane structure.

INTRODUCTION

Photosynthetic membranes of mature chloroplasts disintegrated by detergents and separated by polyacrylamide gel electrophoresis exhibit two pigment-protein complexes as well as redox carriers and enzymes of light-driven electron transfer and photophosphorylation. It is well established that some of the components are attached to the membrane surface while other components are assumed to be embedded into the hydrophobic core of thylakoid membrane [1, 2].

Abbreviations: Complex I and II, pigment-protein complexes. The terms pigment-protein complex and chlorophyll-protein complex are used synonymically.

On the basis of results achieved by exhaustive extraction of thylakoids with 6 M guanidine · HCl, a rather stable construction was postulated which is believed to be the structural framework of the membrane [3]. This basic membrane, insoluble in urea or guanidine · HCl, represents 55–60 % of the total protein mass of the stroma-free membranes and, besides the two pigment-protein complexes, reveals two other polypeptides not yet characterized as well as traces of some other proteins. To complete these results and to achieve further information concerning the molecular composition of the basic membrane the action of proteolytic enzymes was studied.

MATERIALS AND METHODS

Enzymes. Trypsin (chymotrypsin-free, twice crystallized) and pronase P from *Streptomyces griseus* were purchased from Serva, Heidelberg.

Isolation and purification of thylakoid membranes. Envelope- and stroma-free thylakoid membranes from *Vicia faba* leaves were isolated and purified as described previously [4]. The resulting material corresponds to the Type E chloroplast (Hall's nomenclature) and exhibits swollen thylakoids [5].

Proteolytic digestion of entire membranes. Thylakoid membranes were digested on the basis of 60 μg chlorophyll/ml by incubation in 50 mM Tris · HCl buffer, pH 7.5, at 30 °C for 3 h under constant agitation. The following concentrations of proteases were used: trypsin 50–3000 μg/ml; pronase 100–2000 μg/ml. After centrifugation at 10 000 × *g* for 10 min, the indigestible membrane residues were either extracted twice with 8 M urea in 30 mM sodium borate/HCl buffer, pH 8.9, to remove protease molecules attached to the membranes or washed exhaustively with water. The urea extracted material was dissolved in 1 % sodium dodecyl sulfate sodium borate/HCl buffer, pH 8.9. To inhibit proteolytic activity the water washed material was dissolved in the same buffer additionally containing 8 M urea and 2 % β-mercaptoethanol and incubated in a boiling water bath for 2 min. The extracts were prepared for electrophoresis by centrifugation at 150 000 × *g* for 15 min.

Proteolytic digestion of membrane material following sodium dodecyl sulfate disintegration. On the basis of 60 μg chlorophyll/ml thylakoid membranes were disintegrated with 1 ml 30 mM sodium borate/HCl buffer, pH 8.9, containing 1 % sodium dodecyl sulfate. After dilution with detergent-free buffer up to 0.1 % sodium dodecyl sulfate, trypsin was added to a final concentration of 100 μg/ml. Incubation was performed as described. For electrophoresis the solution was concentrated using an Amicon micro-ultrafiltration system 8 MC.

Gel electrophoresis. Gel electrophoresis was performed in columns 5.8 mm in diameter with 10 % acrylamide (Cyanogum 41) as described previously [3, 4]. The following buffer system was used. Upper buffer: 36 mM sodium borate/HCl, pH 8.0, containing 0.1 % sodium dodecyl sulfate. Gel buffer: 380 mM Tris · HCl, pH 8.9. Lower buffer: 72 mM sodium borate/HCl, pH 8.0. To achieve better electrophoretic resolution in some experiments, urea was incorporated into the gels. In such cases stock solutions also containing 5 M deionized urea were used to prepare the gels. The gels were stained with Coomassie brilliant blue G 250 and scanned with a Zeiβ Schnellphotometer.

To isolate pigment-protein Complex II, dissolved membranes were electrophoresed using a preparative Shandon column. The green Complex II band was

resected from the gel with a razor blade and homogenized with 30 mM sodium borate/HCl buffer using a Bühler homogenizer. Sodium dodecyl sulfate was added to a final concentration of 1.0 %. After elution and centrifugation the extract was concentrated and prepared for analytical scale electrophoresis.

RESULTS

Proteolytic digestion by trypsin of entire thylakoid membranes

Envelope- and stroma-free thylakoid membranes swollen by hypotonic shock were digested with 100 μg/ml trypsin at 30 °C for 3 h on the basis of 60 μg/ml chlorophyll. The indigestible residue was washed with water to remove most of the trypsin. To inhibit the activity of trypsin attached to membrane and not removable by water

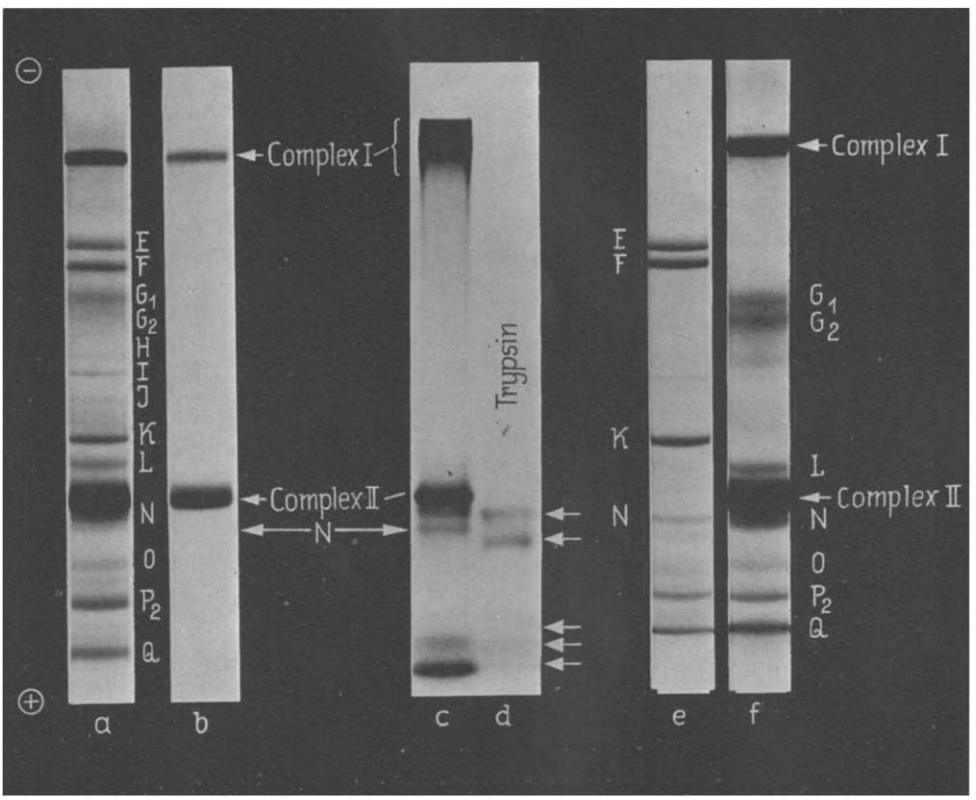

Fig. 1. Effect of trypsin on the polypeptide composition of thylakoid membranes. Envelope- and stroma-free membranes were incubated with 100 μg/ml trypsin for 3 h. Gel a represents the polypeptide pattern of untreated membranes electrophorized on 10 % polyacrylamide gel. Gel b = polypeptide pattern of the trypsin indigestible residue. The membrane attached trypsin was removed by extraction with 8 M urea. Gel c = polypeptide pattern of the trypsin indigestible residue obtained after repeated washings with water. The remaining proteolytic activity was inhibited by incubation in a boiling water bath for 2 min in the presence of 1 % sodium dodecyl sulfate, 8 M urea and 2 % mercaptoethanol. Gel d = polypeptide pattern of the trypsin preparation treated in the same way as described for c. Gel e = polypeptides removable by 8 M urea extraction and electrophorized on 9 % polyacrylamide gels. Gel f = polypeptide pattern of the residue not dissolvable in 8 M urea.

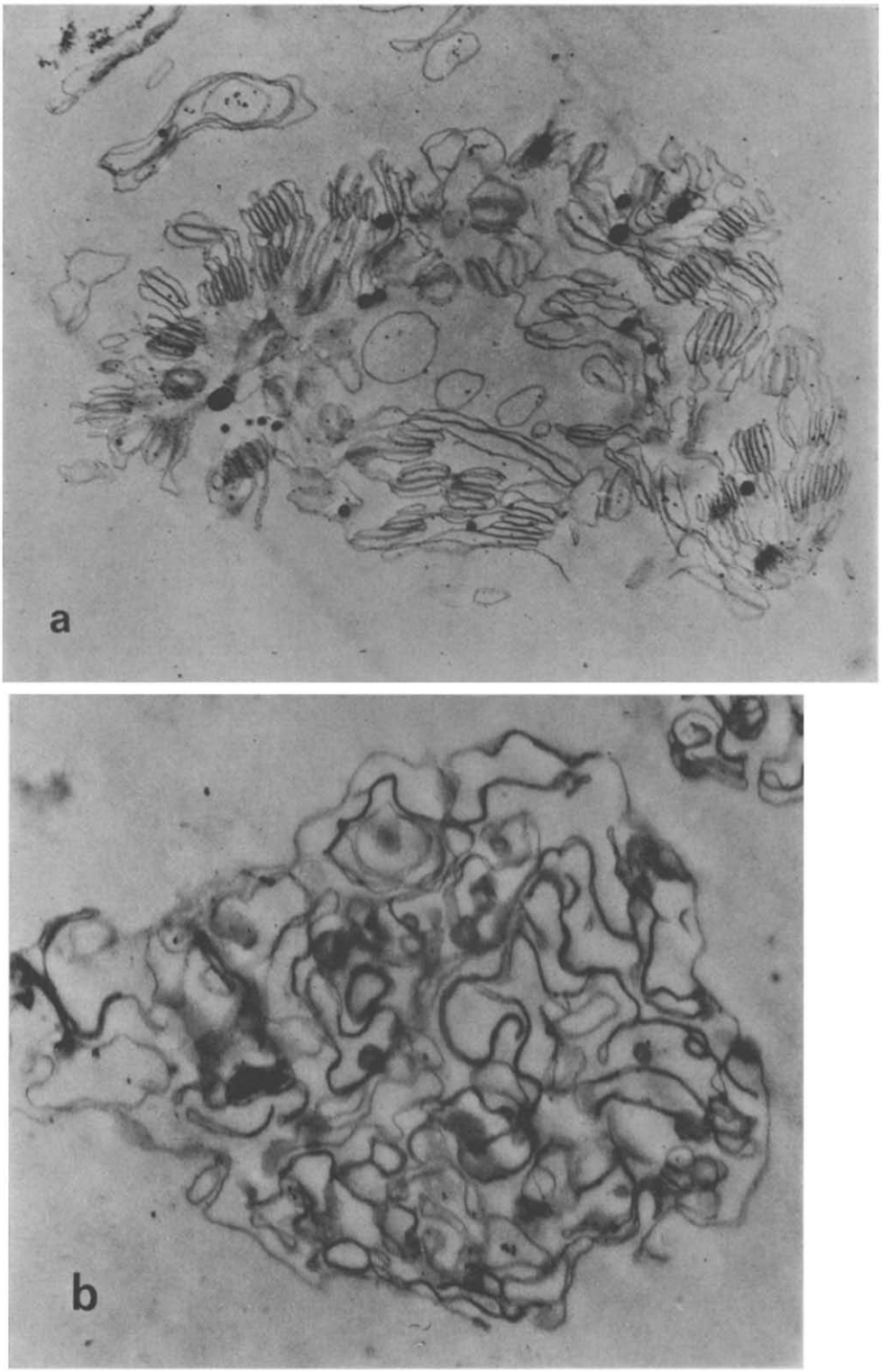

Fig. 2. See opposite page for legend.

washings, the residue was disintegrated with sodium borate/HCl buffer, pH 8.9, containing 1 % sodium dodecyl sulfate and 8 M urea. Mercaptoethanol was added

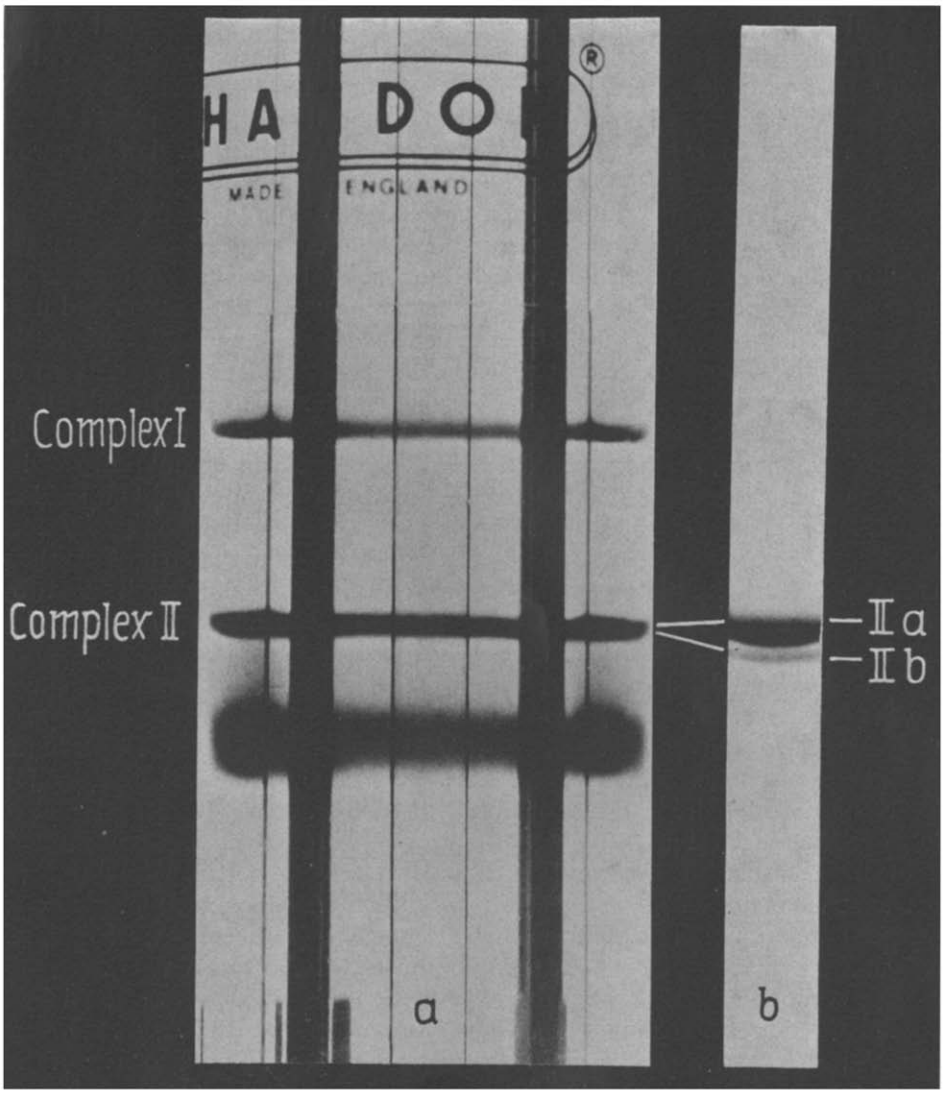

Fig. 3. Re-electrophoresis of isolated pigment-protein Complex II in polyacrylamide gels containing urea. Thylakoid membranes were treated with 6 M guanidine · HCl to remove the associated proteins. After preparative electrophoresis of the sodium dodecyl sulfate disintegrated residue the Complex II band was resected from the gel and homogenized. The eluted Complex II was concentrated and re-electrophorized. In polyacrylamide gel containing 5 M urea Complex II protein splits into two bands which very probably represent two different polypeptides.

Fig. 2. Electron microscopic interpretation of thylakoid membranes incubated with buffer (a) and buffer plus trypsin (b) for 3 h at 30 °C. Membrane attached trypsin was removed by two washings with 8 M urea. Although most of the membrane polypeptides are being removed (Fig. 1) the membrane character of the material is preserved.

to a final concentration of 2 % prior to heating of the material for 2 min in a boiling water bath.

Gel c in Fig. 1 shows the polypeptide pattern of the indigestible residue as compared with trypsin treated in the same way (Gel d) and the untreated control (Gel a). Although some of the polypeptides of the trypsin preparation are found in the lower part of the gel, the electropherogram illustrates that with the exception of the pigment-protein complexes and a polypeptide termed N all the other membrane proteins are digested by the action of this protease. The intensive band of gel c with the highest electrophoretic mobility may represent polypeptide fragments.

Since heating of the disintegrated membranes results in an aggregation of Complex I polypeptides, in some experiments membrane attached trypsin was removed by treatment with 8 M urea prior to sodium dodecyl sulfate disintegration. The polypeptide pattern of the membrane residue surviving trypsination and urea treatment is shown in gel b Fig. 1. Under these conditions, only the pigment-protein complexes and polypeptide N (not visible in the photograph) are constituents of the polypeptide pattern supporting the conclusions that all the other membrane proteins are digestible by trypsin. For comparison, the action of urea without preceding trypsinization is given in gels e and f. Gel e illustrates the membrane polypeptides removeable or partly removable by 8 M urea and gel f the urea resistant residue.

Prolonged incubation or higher trypsin concentration did not greatly alter these results. Electronmicroscopic studies of the residue indigestible by trypsin show the membrane character to be preserved (Fig. 2). However, removing the lipids by treatment with acetone results in complete digestibility of all membrane proteins including the protein moieties of the pigment-protein complexes.

To improve the degree of electrophoretic resolution, urea was incorporated into the polyacrylamide gels. In contrast to Complex I, all chlorophyll molecules were released from the protein moiety of Complex II during electrophoresis in the presence of 5 M urea. Similar results were obtained by Scott and Gregory [6]. Furthermore, under the influence of urea two bands appeared in the position of Complex II termed IIa and IIb (Fig. 3b). According to preliminary experiments it can be assumed that the two bands represent two different polypeptides forming a common band in normal gel systems (10 % acrylamide). This conclusion is supported by results obtained with mutants lacking chlorophyll *b* (Machold, O., Meister, A., Sagromsky, H., Hoyer-Hansen, G. and v. Wettstein, D., in preparation).

Electrophoresis of the trypsin indigestible residue with polyacrylamide gels containing 5 M urea also exhibits the two polypeptides IIa and IIb (Fig. 4). However, as compared with untreated material the components were found to have higher electrophoretic mobility. A possible interpretation of this effect is that small sections were separated from the chains of the two polypeptides by the action of trypsin. Under the supposition that Complex II is an intrinsic membrane particle it may be concluded from the results obtained with trypsin that parts of the complex protrude through the lipid matrix enabling contact with the enzyme.

Proteolytic digestion by trypsin of sodium dodecyl sulfate disintegrated membrane material

It is well established that small concentrations of sodium dodecyl sulfate disintegrate the molecular organization of thylakoid membranes while the native struc-

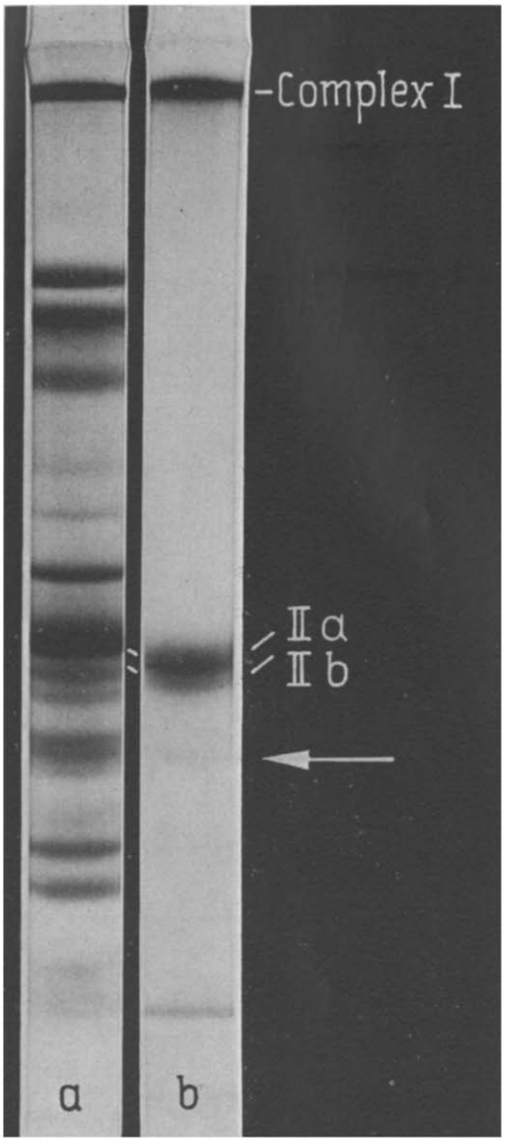

Fig. 4. Electrophoresis of trypsin incubated thylakoid membranes in polyacrylamide gels containing 5 M urea. The material was prepared as described in the legend of Fig. 1. Separation of trypsinized membranes in the presence of 5 M urea yields also two bands in the position of Complex II as compared with the control (Fig. 3 gel b and Fig. 4 gel a). Furthermore, gel b shows the electrophoretic mobility of both components of Complex II to be influenced by the action of trypsin. The arrow marks an additional weak band not digested by trypsin and not yet characterized.

ture of the pigment-protein complexes is being preserved for a rather long time [3, 7]. It is also documented that the proteolytic activity of trypsin is not reduced by small concentrations of sodium dodecyl sulfate [8, 9]. This fact was used to study the action of trypsin on thylakoid membranes disintegrated with 1 % sodium dodecyl sulfate and diluted by buffer up to 0.1 %.

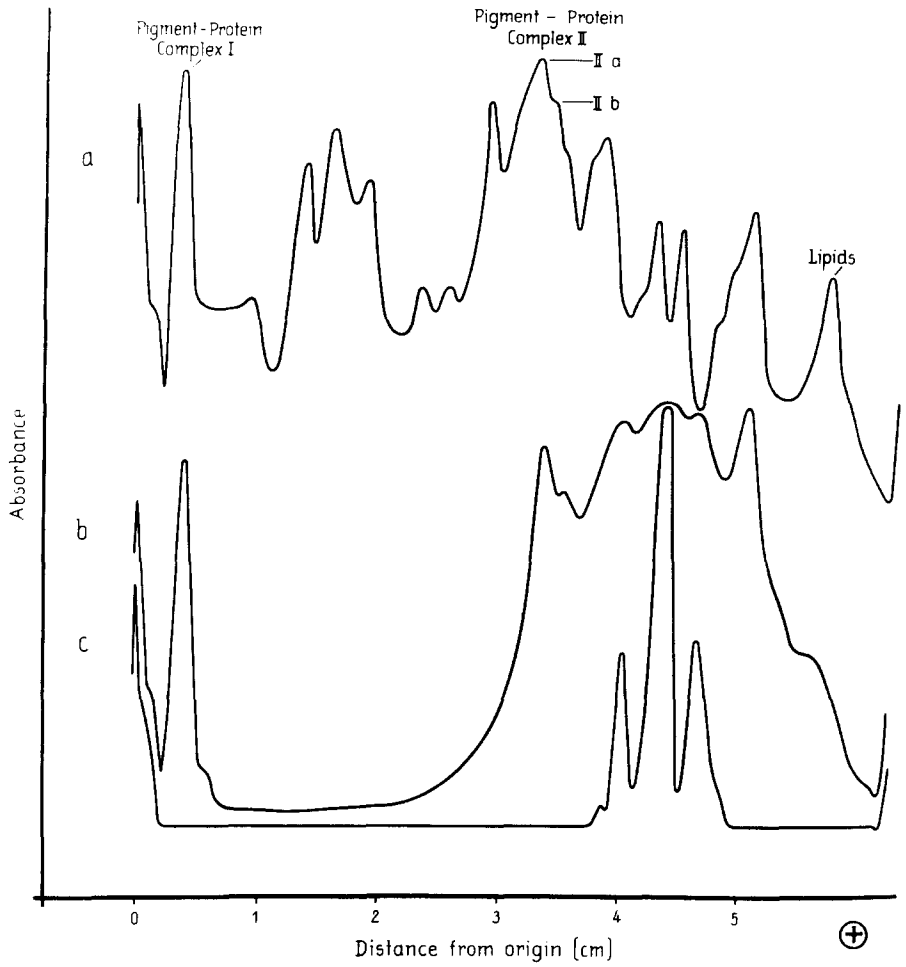

Fig. 5. Effect of trypsin on the polypeptides of thylakoid membranes being disintegrated with 1 % sodium dodecyl sulfate prior to trypsinization. Disintegrated membranes were incubated with 100 μg/ml trypsin for 3 h and separated electrophoretically. Curve a illustrates the densitometric tracings of the polypeptide pattern of the untreated sample. Incubation with trypsin of the disintegrated material in the presence of sodium dodecyl sulfate shows that, with the exception of the pigment-protein complexes, all the other membrane polypeptides were digested (curve b). Curve c exhibits the polypeptide pattern of trypsin. In contrast to Fig. 1 (gel d) mercaptoethanol was not applied to disrupt disulfide bonds.

As shown in Fig. 5 trypsin digests all thylakoid polypeptides localized in the position between the two pigment-protein complexes on the polyacrylamide gel. Since the polypeptide bands of trypsin are found in the lower part of the gel this section is difficult to interpret. In spite of this restriction it may be concluded that, with the exception of the pigment-protein complexes, all proteins of the thylakoid membrane were completely digested in the presence of sodium dodecyl sulfate. These results raise questions as to the stability of the complexes against trypsinization. Since under the specific conditions of the experiments the lipid matrix was disinte-

grated prior to incubation with trypsin, the lipid bilayer may be of subordinate importance in complex protection. It may rather be assumed that the native conformation, which is the result of protein-pigment interactions, is responsible for the stability of the complexes.

Proteolytic digestion by pronase of entire thylakoid membranes

In contrast to trypsin, incubation of thylakoid membranes with 100 μg/ml pronase cause complete digestion of all membrane polypeptides including the protein moieties of the pigment-protein complexes (Fig. 6). As a result of 1 h pronase incubation additional pigmented bands appeared in the patterns. The band termed pigment-protein Complex I_p (Fig. 6) was found by derivative spectroscopy to exhibit Complex I absorption maxima but nevertheless, to have a higher electrophoretic mobility as

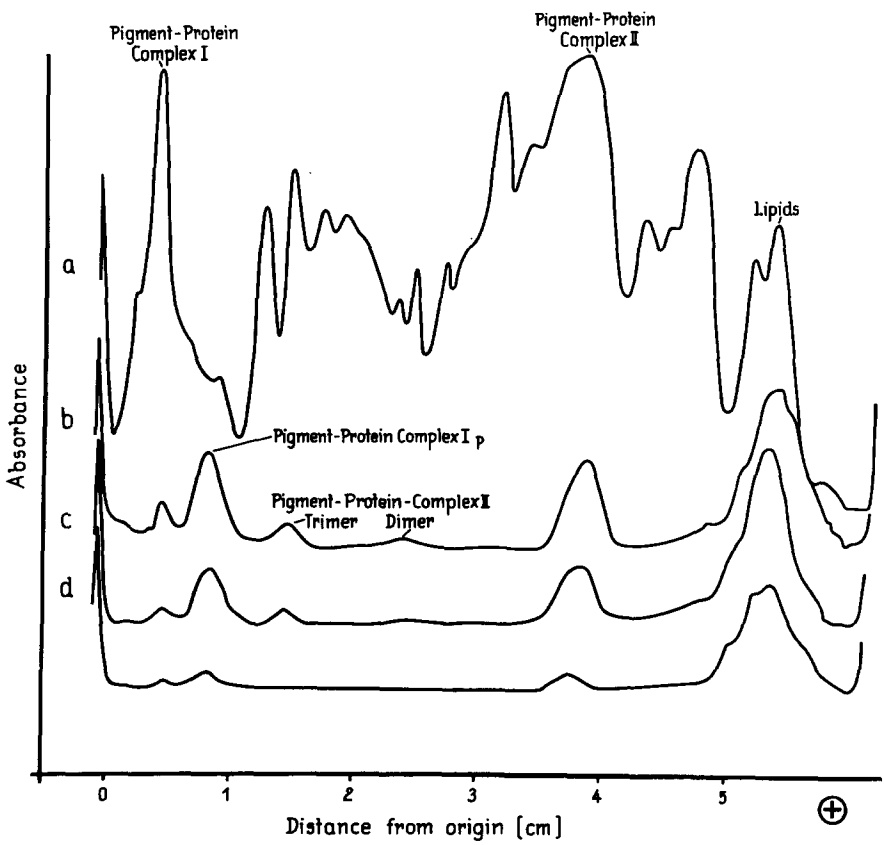

Fig. 6. Effect of pronase on thylakoid membranes. Thylakoid membranes were incubated with 100 μg/ml pronase P for 1 (curve b), 3 (curve c) and 8 (curve d) h. Pronase molecules attached to the indigestible residue were removed by extraction with 8 M urea. Densitometric tracings of polypeptide patterns obtained after disintegration of the indigestable residue and followed by electrophoresis shows that all membrane proteins including the pigment-protein complexes can be digested by pronase. Pigment-protein Complex I_p represents a coloured band with the specific absorption maxima of Complex I but higher electrophoretic mobility caused by the influence of pronase.

compared to Complex I. The other additional pigment bands were identified as dimers and trimers of pigment-protein Complex II. After 8 h of incubation the indigestible residue yielded only traces of protein which completely disappeared after 14 h of incubation. Electron-microscopic studies of the material which still contained all the pigments showed an amorphous mass without visible membrane structures. These results support the suggestion that the pigment-protein complexes are indispensable in maintaining membrane structure.

DISCUSSION

It is well documented that proteases are able to attack biological membranes in vitro. Controlled proteolysis proved to be a useful counterpart to labelling experiments in studies of organization of proteins in human red blood cell membranes [10–12] and other membrane types [13, 14]. With respect to chloroplast thylakoid membranes, attention was focused on finding out an inhibitory effect of proteases on specific functions of the photosynthetic apparatus (15–20) without considering its influence on the molecular architecture.

The present experiments were carried out with the object of completing previous results achieved by application of chaotropic agents [3]. The results of these studies showed most of the membrane proteins to be attached by polar or weak hydrophobic interactions to the surface of a rather stable basic construction. Similar conclusions can be drawn from the experiments described in this paper.

With the exception of the pigment-protein complexes and small amounts of a polypeptide not yet characterized trypsinization removed all the other proteins from the basic membrane. In comparison with guanidine · HCl, two polypeptides bound by strong hydrophobic interactions were also released by trypsin. Chymotrypsin was found, in experiments not described in the present paper, to have the same effect. From these results it can be concluded that the protein components of electron transfer and photophosphorylation including the two hydrophobic bound polypeptides previously termed G_1 and G_2 [3] and representing about half of the protein mass are extrinsic in the sense that they are removable without disruption of the basic membrane structure. Although the positions of the extrinsic or associated proteins are not exactly known most of the components may be assumed to be localized at or near the surface. This is in conformity with findings of other authors [21–23].

Extractibility by perturbants of about half of the protein mass has also been reported from other types of membranes, as e.g. for erythrocytes. However, in contrast to thylakoids some red cell membrane proteins removable by specific denaturants were found not to be digestible by trypsin [10].

Concerning the localization of the pigment-protein complexes it has been suggested from results obtained previously that these components are inserted partially or entirely into the hydrophobic core of the membranes [3]. The present findings do not contradict this assumption. However, since trypsin was found to influence the electrophoretic mobility of Complex II it should be expected that at least parts of the protein moieties protrude the surface of the lipid matrix.

In this connection, the stability against trypsinization of the pigment-protein complexes is of interest. Since the complexes are being either not digested or only partially digested by trypsin whether or not the membrane structure was disrupted

by sodium dodecyl sulfate, their native conformation due to pigment-protein interactions must be an important factor.

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