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PARTITION ZONE PENETRATION BY CHYMOTRYPSIN, AND THE LOCALIZATION OF THE CHLOROPLAST FLAVOPROTEIN AND PHOTOSYSTEM II

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Summary

1. Chymotrypsin treatment of chloroplast membranes inactivates Photosystem II. The inactivation is higher when the activity is measured under low intensity actinic light, suggesting that primary photochemistry is preferentially inactivated.

2. Membrane stacking induced by Mg^{2+} protects Photosystem II against chymotrypsin inactivation. When the membranes are irreversibly unstacked by brief treatment with trypsin, Mg^{2+} protection against chymotrypsin inactivation of Photosystem II is abolished.

3. The kinetics of inactivation by chymotrypsin of Photosystem II indicates that membrane stacking slows down, but does not prevent, the access of chymotrypsin to Photosystem II, which is mostly located within the partition zones.

4. It is concluded that a partition gap exists between stacked membranes of about 45 Å, the size of the chymotrypsin molecule.

5. The kinetics of inhibition of the chloroplast flavoprotein, ferredoxin-NADP reductase, by its specific antibody is not affected by membrane stacking. This indicates that this enzyme is located outside the partition zones.

Introduction

The photosynthetic membranes of the chloroplast are arranged into stacked regions of high membrane density separated by unstacked regions of much lower membrane density. It is generally considered, on the basis of electron

microscope work, that adjacent membranes in stacked regions are in some kind of physical contact one with another [1,2]. However Nir and Pease [3] consider that this conclusion, with respect to transmission electron microscope work, is incorrect and due to artifacts produced during preparation of the membranes for microscopy. With a glutaraldehyde-urea embedding technique which avoids the use of lipid and chlorophyll solvents, they have obtained results which they claim are indicative of a partition gap of about 40 Å between the adjacent thylakoids of membrane stacks. As well over 50% of the thylakoid surface is usually in the partition zone, this is a problem of considerable importance from the point of view of the restrictions it may impose on the positioning of at least some of the electron transport and phosphorylation components.

The problem of the distribution of the various electron transport components along the thylakoid membranes has been examined mainly from the point of view of certain components being concentrated inside or outside the grana stacks (for recent review, see Ref. 4).

The techniques most commonly employed for these studies, detergent or French Press fractionation, do not permit the localization of components with respect to the partition zones. However, electron microscope studies have permitted Miller and Staehlin [5] and Murakami and Kunieda [6] to suggest that the coupling factor complex is mainly located outside the partition regions. Recently Armond and Arntzen [7] and Staehlin [8] have suggested that up to 80% of Photosystem II is located in the partition zones, based on studies of freeze fracture particle distribution.

In order to gain information both on the distribution along the membranes of the various electron transport components and on the degree of tightness of membrane stacking, we compared the accessibility of electron transport components to large molecules in stacked and unstacked membranes. The possibility of comparing the properties of stacked and unstacked membranes in vitro has been opened up by the work of Izawa and Good [9]. These authors first observed the dramatic effect of cations on chloroplast membrane structure. Incubation of chloroplasts in low-salt medium resulted in the unstacking of grana that could be reversed upon readdition of high concentrations of monovalent cations or low concentrations of divalents. These effects have since been confirmed by others [10—13]. Thus, divalent cations in the range of 1—5 mM and monovalent cations in the range of 50—150 mM stimulate membrane stacking. A rather similar cation concentration requirement has also been demonstrated for increasing the variable component of chlorophyll fluorescence, in a reversible fashion [14—16]. We have demonstrated [17,18] that brief treatment of chloroplast membranes with the proteolytic enzyme trypsin destroys irreversibly the ability to form membrane stacks upon cation addition. Furthermore, the cation-sensitive fluorescence was shown to be an accurate indicator of the degree of stacking [19]; a conclusion in accord with that of Argyroudi-Akoyunoglou and Akoyunoglou [20] using different methods. In the work presented here we demonstrate that chymotrypsin treatment inactivates Photosystem II activity and that this inactivation is slowed down initially by as much as 50—60%, but is not prevented, by membrane stacking. Using a similar rationale we have examined the localization of the ferredoxin-NADP

reductase (EC 1.6.99.4) by examining the kinetics of antibody inhibition in stacked and unstacked membrane preparations. No differences are observed which leads us to conclude that this enzyme is located outside the partition zones, a conclusion somewhat different to that of Schmid and Radunz [21].

Materials and Methods

Chloroplasts were prepared from freshly harvested spinach leaves by a brief homogenisation in an extraction buffer comprising 30 mM Tricine (pH 8.0), 10 mM NaCl, 0.4 M sucrose and sometimes 2.5 mM $MgCl_2$, depending on the experiment. The extract was centrifuged at 1500 × *g* for 5 min and the chloroplast pellet was resuspended in the same buffer. This buffer also served as the reaction medium, with the omission of sucrose. Chloroplast preparation was performed at 0 to 4°C.

For electron microscopy, chloroplast samples were fixed for 1 h in 2% glutaraldehyde in the same buffer as that of the incubation. Samples were then washed several times in the same buffer ($\pm Mg^{2+}$), and postfixed for 1 h in 1% osmium tetroxide in phosphate buffer (pH 7.5, $\pm Mg^{2+}$). After postfixation the samples were embedded in agar and dehydrated. During dehydration the preparations were treated for 3 h with 75% ethanol saturated with uranyl acetate. After dehydration they were embedded in epon. Ultrathin sections were stained with lead citrate.

Electron transport from water to dibromothymoquinone (15 μM) was measured as O_2 evolution with an oxygen electrode in the presence of ferricyanide (1 mM). The chlorophyll concentration was always 20 μg/ml.

α-Chymotrypsin (Type 1-S) was purchased from the Sigma Chemical Co.

Fluorescence measurements were made with a Perkin-Elmer MPF-3 spectrofluorimeter with an excitation wavelength of 440 nm and an emission wavelength of 685 nm. The excitation light intensity was about 1500 ergs · s^{-1} · cm^{-2}, sufficient to maintain Q in a fully reduced state in the absence of an electron acceptor.

The NADP diaphorase activity of the ferredoxin-NADP reductase was measured essentially as previously described [22]. The chloroplast membranes (2–6 μg of chlorophyll/ml) were incubated with the following: 30 mM Tricine (pH 8.0), 10 mM NaCl, 0.8 mM ferricyanide, 0.5 mM NADP, 5 mM glucose 6-phosphate, and glucose-6-phosphate dehydrogenase in large excess (added as the last addition, to generate NADPH). The rate of ferricyanide reduction was followed at 420 nm. Alternatively, the diaphorase activity was measured with the system ferredoxin-cytochrome *c* as the electron acceptor. In this case, ferricyanide was omitted and ferredoxin (22.1 μM) and cytochrome *c* (110 μM) were added; the rate of reduction of cytochrome was followed at 550 nm.

For the preparation of antibody against the ferredoxin-NADP reductase, 1 mg of the enzyme, obtained in the pure state according to Forti and Sturani [22] was injected into rabbits subcutaneously with complete Freund's adjuvant. After one week, a second injection, of 1.3 mg of enzyme, was given with incomplete Freund's adjuvant, subcutaneously. After 10 days, a booster injection of 2.2 mg of enzyme was given intravenously. The rabbits were periodically bled, the serum was collected and the γ-globulin fraction purified by

ammonium sulfate fractionation [23]. The same purification procedure was applied to the non-immune sera. No effect whatsoever was observed with non-immune γ-globulins. One unit of antibody was defined as the amount which inactivates one unit of diaphorase activity, as measured by the ferricyanide assay (1 unit: 1 nequiv. per min).

NADP photoreduction was measured in buffer of 30 mM Tricine pH 8.0/10 mM NaCl/0.5 NADP/5.1 μM ferredoxin/5 μM Gramicidin/19.5 μg/ml chlorophyll. The chloroplasts were preincubated for 5 min with or without antibody, as indicated. The absorbance at 340 nm was then measured before and after exposure of the reaction cuvette to saturating white light for 1 min. NADP reduction was calculated from the absorbance change at 340 nm.

Results

In Table I it can be seen that chymotrypsin inhibits the Photosystem II reduction of dibromothymoquinone with water as electron donor. The inhibition was higher when electron transport was measured at relatively low light intensities, which is taken to indicate that chymotrypsin primarily lowers Photosystem II efficiency, and that digestion is therefore presumably very close to the photosystem itself.

In Table II are data showing the typical effect of Mg^{2+}, present during a 3-min incubation before chymotrypsin addition and present during protease digestion, on the subsequent photochemical activity of Photosystem II. In the absence of Mg^{2+} (unstacked chloroplasts, [9]; see also Fig. 1) chymotrypsin reduced the activity by 54%, while in the presence of Mg^{2+} (stacked chloroplasts [9]; see also Fig. 1) this inactivation was only 24%. For two reasons this effect is attributed to a protective effect of Mg^{2+} against chymotrypsin attack. The final concentration of Mg^{2+} (i.e., during the measurement of Photosystem II activity) was equal at 10 mM in both chloroplast samples and in the absence of chymotrypsin no difference is observed between chloroplasts to which Mg^{2+} was added just before electron transport measurements or at the beginning of the 25°C incubation period.

We have observed that chymotrypsin treatment of stacked membranes is able to bring about a slow destacking. However this occurs only at high chymotrypsin concentration and after long digestion times, and seems to be reflected

TABLE I

EFFECT OF CHYMOTRYPSIN DIGESTION ON ELECTRON TRANSPORT FROM WATER TO THE DIBROMOTHYMOQUINONE-FERRICYANIDE SYSTEM

Chymotrypsin was added at a concentration of 40 μg/ml for 2.5 min before electron transport was measured. A Corning 4-96 filter was used. The high intensity was 315 000 ergs · cm^{-2} · s^{-1}, while the low intensity was 52 000 ergs · cm^{-2} · s^{-1}. Numbers are nequiv/μg chlorophyll per h.

	High light		Low light	
	Control	Treated	Control	Treated
Rate	197	138	79	32
Percent inhibition	—	30	—	60

TABLE II

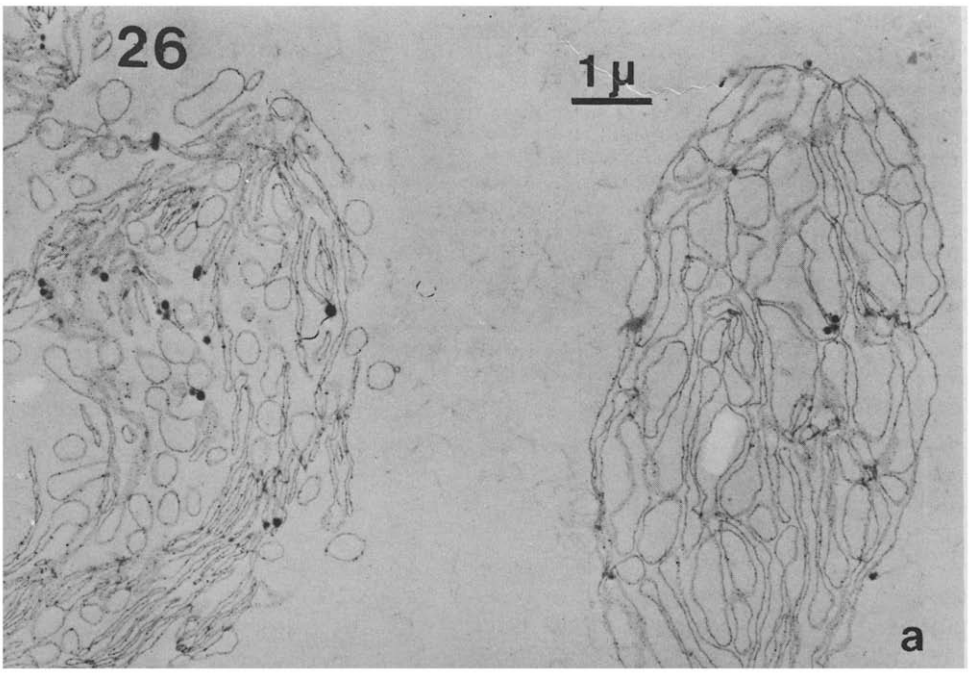
MAGNESIUM ION PROTECTION AGAINST CHYMOTRYPSIN DIGESTION OF PHOTOSYSTEM II ACTIVITY

Chloroplasts (20 μg/ml) were incubated at 25°C for 3 min in the presence or absence of $MgCl_2$ (5 mM). Chymotrypsin was then added (20 μg/ml) where indicated, and the incubation was continued for 4 min. At the end of this period (7 min since the beginning of incubation at 25°C), $MgCl_2$ was added to bring the concentration in all cases to 10 mM, followed by addition of dibromothymoquinone and ferricyanide. After 2 min (6 min from chymotrypsin addition) illumination ($2 \cdot 10^5$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1}, Corning 4-96 filter) was started. Electron transport was measured for 1 min. Rates are nequiv./μg chlorophyll per h. In the same experiment samples were taken for fluorescence measurements (given in arbitrary units in the table) and electron microscopy, immediately before the addition of chymotrypsin and immediately before illumination. For the electron microscope pictures see Fig. 1.

Additions:	None	$MgCl_2$	Chymotrypsin	$MgCl_2$ + chymotrypsin
Electron transport	134	131	62	102
Fluorescence	26	57	20	51

by the cation sensitive fluorescence (Fig. 1). We have therefore routinely measured cation stimulation of fluorescence yield as an indicator of the state of membrane stacking during our experiments with chymotrypsin. In Table II it is clear that the effect of Mg^{2+} on fluorescence yield was approximately intact after chymotrypsin (20 μg/ml) treatment for 6 min, which is taken to indicate a high level of stacking in Mg^{2+}-treated samples. This conclusion has been directly confirmed for that particular experiment (Fig. 1c).

While these data are consistent with the idea of membrane stacking impeding the access of chymotrypsin to the partition zones, we felt that a more conclusive demonstration was desirable. To this end we pretreated chloroplasts for different periods of time with trypsin, a treatment known to cause membrane unstacking and which can be determined by measuring the cation-sensitive chlorophyll fluorescence [17,19]. The data from several such experiments are combined in Fig. 2, where it can be seen that the protective effect of $MgCl_2$ against chymotrypsin digestion was substantial only when membranes were fully stacked. After 30 or 60 s of trypsin treatment the degree of membrane stacking decreased markedly, and the protection against chymotrypsin decreased in an approximately parallel fashion. In order to decide whether stacking prevents chymotrypsin access to Photosystem II, or only kinetically impedes this access, we performed the experiments shown in Fig. 3. It can be seen that the extensive chymotrypsinization led to an approximately 40—50% reduction in membrane stacking (as judged by fluorescence measurements), but this remained fairly constant from 3 to 12 min of chymotrypsin treatment. The protection offered to Photosystem II activity by membrane stacking is seen to disappear with increasing digestion time, and after 9—12 min both stacked and unstacked chloroplasts displayed a similar degree of inhibition. These results were obtained with chloroplasts stored in the ice prior to experimental manipulation in the absence of Mg^{2+}, and which were stacked during a 3 min incubation with Mg^{2+} before chymotrypsin treatment. We have obtained similar results also with chloroplasts extracted in the presence of 2.5 mM Mg^{2+} and then stored in ice in either the presence or absence of Mg^{2+} for 1 h before start-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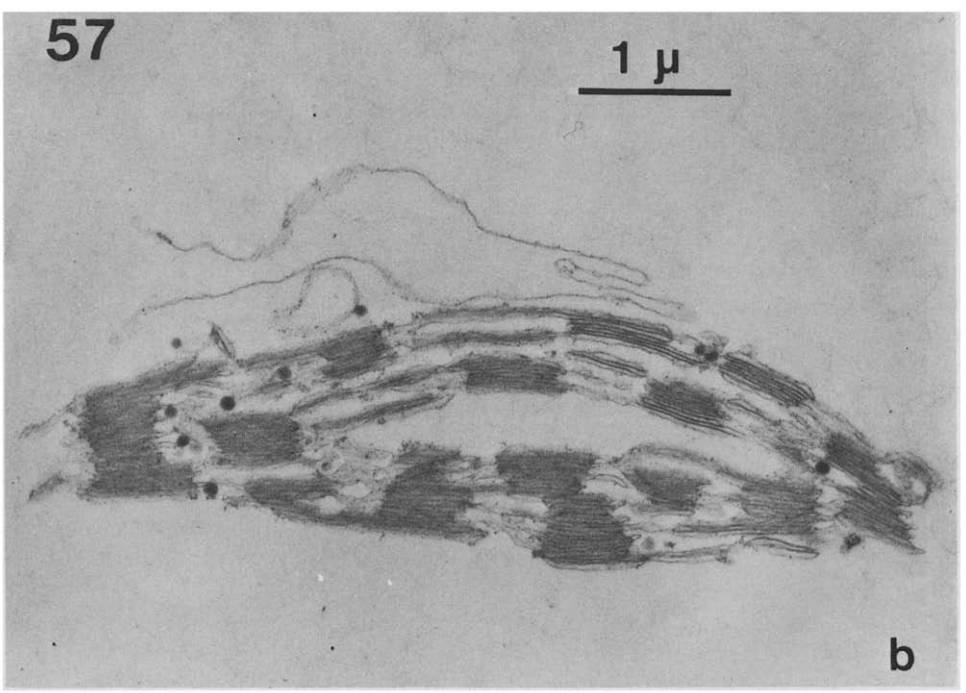
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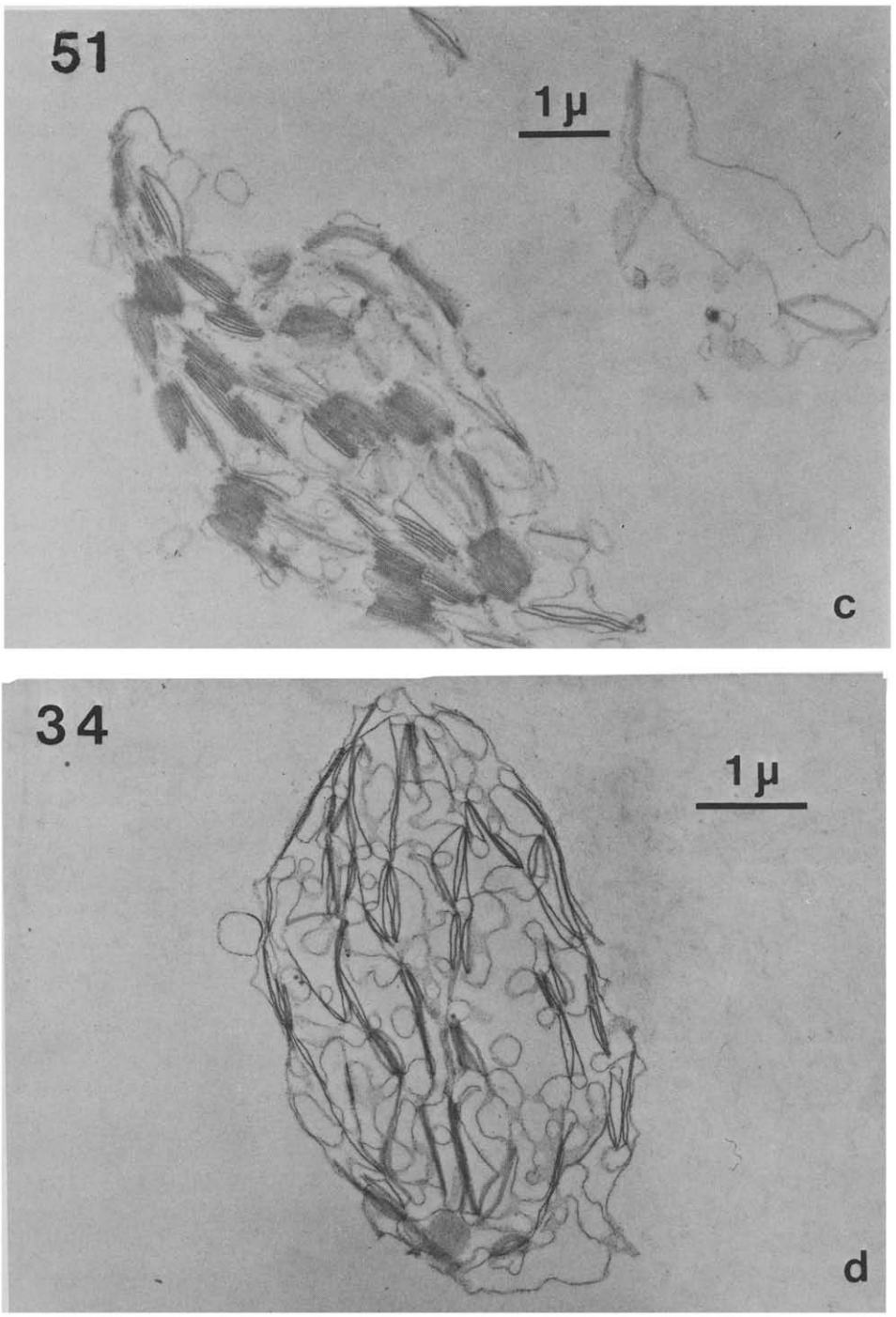

Fig. 1. Electron micrograph pictures of the effect of chymotrypsin on membrane stacking in chloroplasts. a, Control without further additions. b, Control + 5 mM $MgCl_2$. c, Control + $MgCl_2$ + chymotrypsin (20 μg/ml, 6 min). d, Control + $MgCl_2$ + chymotrypsin (80 μg/ml; 17 min). The experimental format is that of Table II. The numbers on the micrographs represent the fluorescence values of the particular samples.

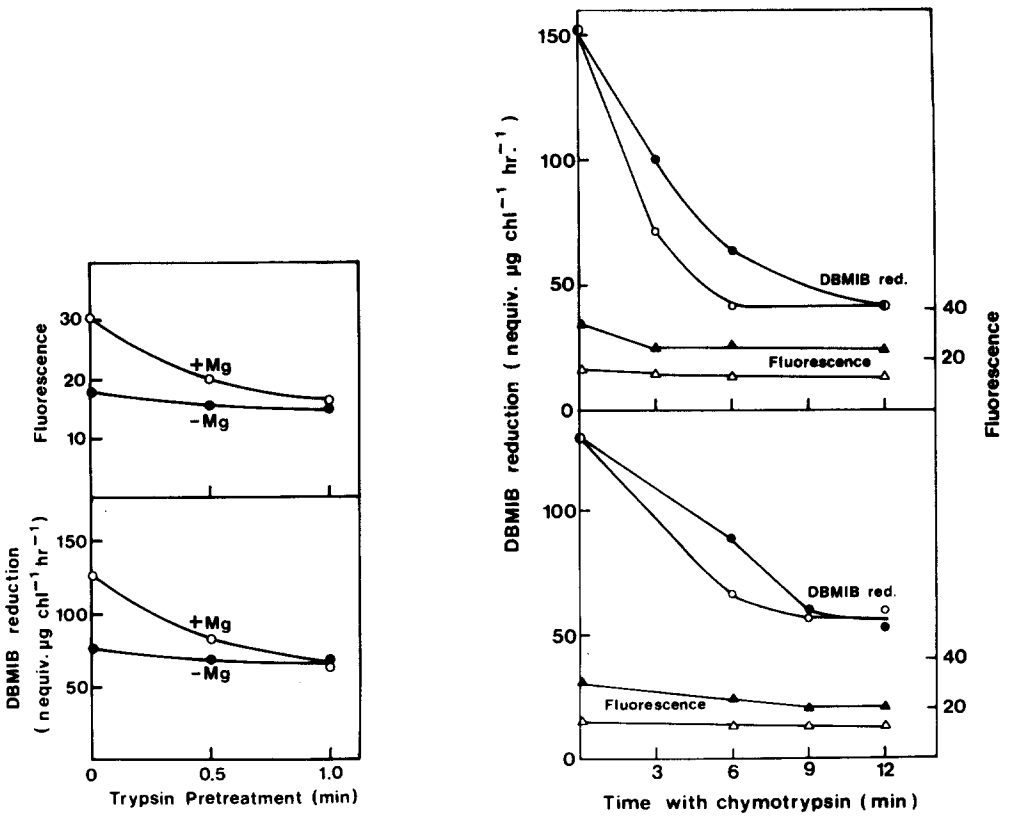

Fig. 2. Effect of unstacking of chloroplast membranes by a trypsin pretreatment on the subsequent protection offered by $MgCl_2$ against chymotrypsin inactivation of Photosystem II. Chloroplasts were initially treated with trypsin (2 µg/ml) for 0, 30 and 60 s when a tenfold excess of trypsin inhibitor was added. This was followed by a 3 min incubation period with or without $MgCl_2$ (5 mM), when 20 µg/ml chymotrypsin was added. 4 min later, $MgCl_2$ was added to a final concentration of 10 mM, and the dibromothymoquinone/ferricyanide acceptor system was added. 6 min after chymotrypsin addition, illumination was commenced (200 000 ergs · cm^{-2} · s^{-1}, Corning 4-96 filter) and electron transport was measured for 1 min. Samples were taken for fluorescence measurement just before chymotrypsin addition to estimate the effect of trypsin in preventing membrane stacking. Electron transport activities are nequiv./µg chlorophyll per h.

Fig. 3. Two experiments showing the effect of chymotrypsin digestion on Photosystem II activity and the cation stimulated fluorescence. The experiment was performed as in Table II, except that chymotrypsin was 80 µg/ml and the length of chymotrypsin digestion was variable. Solid symbols: $+MgCl_2$ before and during chymotrypsin treatment. Open symbols: $-MgCl_2$ before and during chymotrypsin treatment. It should be noted that the fluorescence measurements were made at the end of the chymotrypsin treatment, and are thus indicative of the minimum level of thylakoid stacking attained during the experiment.

ing the experiment (see the legend to Fig. 4 for details of this type of chloroplast preparation).

To investigate the possibility that the degree of membrane stacking may in some manner vary during electron transport, in the light, we examined the protection offered by stacking against chymotrypsin inactivation of Photosystem II carried out either in the light or in the dark. It was found that no difference in protection by Mg^{2+} against chymotrypsin is observed whether chymotrypsinization occurs in light or dark in the presence of methyl viologen.

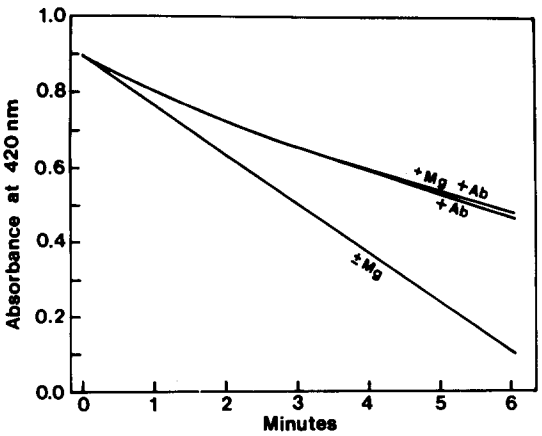

Fig. 4. Kinetics of inhibition by the antibody of NADPH diaphorase activity of chloroplasts. Conditions: see Methods. Ferricyanide was the electron acceptor. Chlorophyll was 4.3 μg/ml. Antibody (the γ-globulin fraction of the serum of immunized rabbits) was 240 units, added at time zero. Chloroplasts were prepared with 2.5 mM $MgCl_2$ in the grinding medium and resuspended in the presence and absence of $MgCl_2$, as indicated. The chloroplasts were kept at 0°C for about 1 h before the experiment.

Thus we conclude that the degree of membrane stacking is not influenced by such factors as electron transport and illumination.

The work presented here confirms earlier conclusions of others concerning the location of the major part of Photosystem II within the partition zones [7]. It also demonstrates that membrane stacking impedes the penetration of macromolecules to sites on the membrane surface within the partition zones. We therefore decided to examine the position of the 'other end' of the electron transport chain, i.e. the ferredoxin-NADP reductase. The method adopted was to examine the kinetics of inhibition by antibodies against this enzyme in stacked and unstacked membranes. To this end we assayed the reductase using its NADPH diaphorase activity, which has the advantage that this reaction is not influenced by $MgCl_2$. Fig. 4 shows that the NADPH diaphorase (with ferricyanide as the acceptor) shows identical kinetics of inhibition by the antibody in the presence and absence of Mg^{2+}, i.e., whether the membranes are stacked or not. The same stituation is seen when ferredoxin and cytochrome *c* are used as the electron acceptor system (Fig. 5). For two reasons, it has not been possible to examine the kinetics of antibody inhibition of this enzyme in stacked and unstacked membranes by measuring the light-induced reduction of NADP. Firstly the reaction itself is sensitive to cations [24]; secondly, the ferrodoxin-NADP reductase cannot be made rate limiting before a substantial inhibition of it by the antibody. However, we have noticed that in the presence or absence of membrane stacking the final level of inhibition by antibody is unaffected. This observation has been made over a wide range of membrane/antibody concentration ratios.

It should be noted (Fig. 6) that when ferricyanide is the electron acceptor, complete inhibition of the diaphorase activity is not observed, while the ferredoxin-cytochrome *c* system is completely inhibited, as is NADP photoreduction (both in the presence and absence of Mg^{2+}) which requires added ferre-

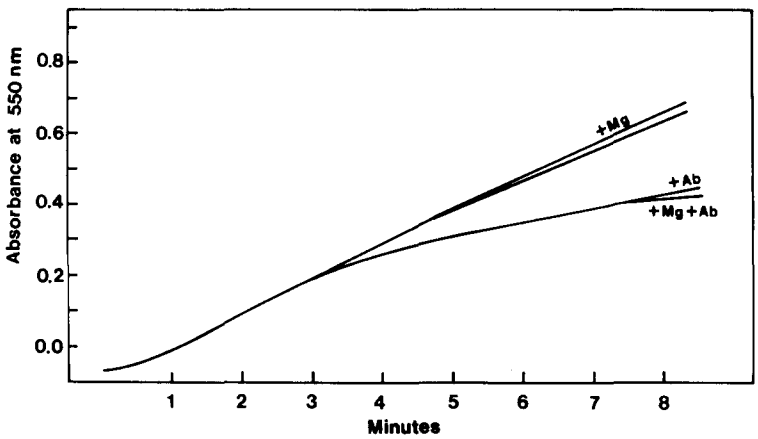

Fig. 5. Kinetics of inhibition by the antibody of NADPH diaphorase activity of chloroplasts. Conditions: see Methods. Cytochrome *c* was the electron acceptor. Chlorophyll was 2.6 μg/ml. Antibody was 60 units added at 3 min.

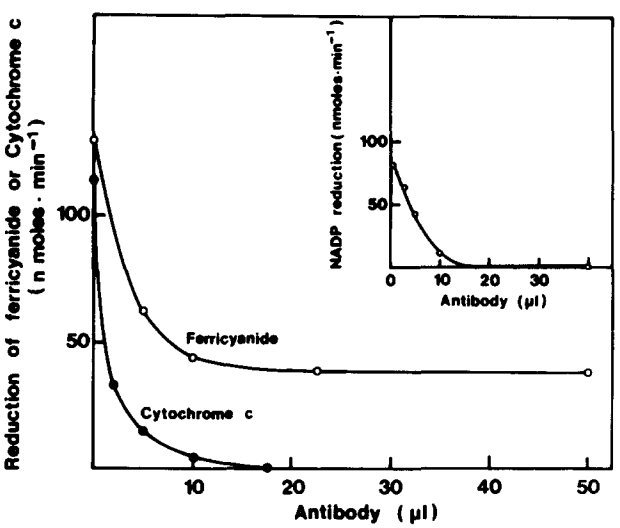

Fig. 6. Inhibition of the different reactions of chloroplast ferredoxin-NADP reductase by the antibody. Conditions: see Methods. The antibody added was of 60 units/μl. Preincubation with antibody was for 5 min in the absence of the electron acceptor system. This time was in excess over that needed to ensure the complete effect of the antibody added. NADP photoreduction was measured in the absence of Mg^{2+}; however, the same inhibition by antibody was observed in the presence of 5 mM $MgCl_2$ (not shown).

doxin. Complete inhibition of all the reactions catalysed by the flavoprotein is observed when the soluble enzyme is treated with the antibody (unpublished data).

Discussion

The above data indicate that chymotrypsin, which inactivates the Photosystem II reduction of dibromothymoquinone-ferricyanide, probably at or very close to the photosystem itself, has a reduced access to the membrane surfaces when

they are stacked by addition of Mg^{2+}. As the final level of inactivation in stacked and unstacked membranes is the same (i.e., after prolonged periods of digestion with the protease), we believe this to indicate that the chymotrypsin can penetrate the partition zones, but access to the stacked membrane surfaces is slowed down. This conclusion is significant with respect to ideas concerning the 'tightness' of the partition zone. Obviously it is not possible to reconcile these data with the idea of an extremely close and stable contact between adjacent stacked membranes. Sufficient space must exist to permit the passage of some chymotrypsin. Thus our data would tend to support the idea of Nir and Pease [3] and the width of 40 Å which they proposed for this 'gap' would be in accordance with our data. It should be pointed out the longest axis of the chymotrypsin molecules is about 45 Å [25]. It would therefore seem possible that suitably sized stromal components could have a relatively free access to the partition gaps.

The observation that access of chymotrypsin to Photosystem II is impeded by membrane stacking indicates that Photosystem II is at least partly in the partition zones. Though these data do not lend themselves to a precise quantization of the proportion of Photosystem II in the partition zones, the degree of stacking-induced protection observed is often of the order of 50—60%. This must be a minimum number for the amount of Photosystem II in the partition zones, because as we have pointed out above, the chymotrypsin is able to penetrate these zones. On the basis of the identification of the large freeze-fracture particles as Photosystem II units, both Staehlin [8] and Armond and Arntzen [7] estimate around 80% of Photosystem II to be in the partition zones.

It is also demonstrated that an antibody preparation against the ferredoxin-NADP reductase of spinach chloroplasts is able to inhibit the activity of this enzyme in a manner which is not influenced by membrane stacking. As the antibody molecules are of molecular weight and dimension greatly in excess of the chymotrypsin, it is difficult to escape from the conclusion that the flavoprotein is all external to the partition zones. Thus it is probably distributed on the stroma lamellae and also on the end membranes of the grana stacks. This conclusion is at variance with that of Schmid and Radunz [21] who estimated the maximal amount of NADP-reductase inhibited by antibody in mutants of tobacco with different degrees of membrane stacking. They noticed an inverse correlation between membrane stacking and the degree of inhibition by antibody. Also, $MgCl_2$ added at rather high concentrations decreased the extent of antibody reaction, which may also have correlated inversely with membrane stacking, though the latter point was not demonstrated. We are not able at present to explain the differences in results obtained by us working with spinach chloroplasts and by Schmid and Radunz with tobacco, though it does seem possible that the differences in antibody reactivity observed by these workers in the tobacco mutants could have been caused by other factors related to membrane structure, not necessarily related to stacking.

The conclusion that all the ferredoxin-NADP reductase is found outside the grana partition regions and that Photosystem II is mostly located within the partitions, raises the question of how reducing equivalents are transferred to this enzyme.

At present we are unable to comment on the localisation of other electron

carriers, though work is in progress on this point. However, it would seem necessary to invoke the involvement of a mobile electron carrier. This idea is not new, and has already been suggested by Sane et al. [26]. Of known electron carriers the most likely candidate for this role, on the basis of its molecular size, membrane solubility and relatively high concentration, would be plastoquinone. In this respect it is interesting that several different lines of evidence suggest that the intermixing of electrons between different electron transport chains occurs at the level of plastoquinone [27,28]. However before such a role can be assigned with any confidence to plastoquinone we must learn something of the distribution of Photosystem I itself.

In the course of the work on flavoprotein localisation we noticed that for the diaphorase activity when ferricyanide was the electron acceptor, the maximal inhibition obtained with high antibody/membrane ratios was 70—75%. Incomplete inhibition of this reaction has also been observed by others (see Fig. 4B, Ref. 29). However for both the diaphorase activity with ferredoxin-cytochrome *c* as the electron acceptor system, or for the light induced reduction of NADP where added ferredoxin reacted with the flavoprotein, complete inhibition by the antibody was observed in the presence or absence of Mg^{2+}. These differences might be due to the fact that the antibody molecules reacting with the enzyme on the surface of the membranes may protect, by steric hindrance, residual enzyme molecules against antibody. Small electron acceptor molecules such as ferricyanide would have access to these residual active enzyme molecules, while an activity dependent on macromolecules such as ferredoxin is completely inhibited by an excess of antibody.

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