Effects of Trypsin and Cations on Chloroplast Membranes

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ABSTRACT

A mild trypsic digestion of chloroplast membranes eliminates the effects of saturating concentrations of cations (3 to 5 millimolar MgCl₂) on chlorophyll fluorescence yield, membrane stacking, and photosystem II photochemical efficiency in spinach. At the same time, the negative surface potential of the membranes is increased (by trypsic) as revealed by studies with 9-aminoacridine. High concentrations of cations (25 to 100 millimolar MgCl₂) added after trypsic digestion are effective in restoring high fluorescence yields and membrane stacking. High concentrations of cations added after trypsic treatment do not increase the photosystem II efficiency. It is concluded that the "diffuse electrical layer" hypothesis of Barber et al. (Barber J, J Mills, A Love, 1977 FEBS Lett 74: 174-181) satisfactorily explains the effect of trypsic in eliminating the influence of saturating concentrations of cations on chlorophyll fluorescence yield and membrane stacking. However, the effect on photosystem II photochemical efficiency seems to require another mechanism.

It has been known for many years that cations exert powerful effects on chloroplast structure and electron transport activities. Principal among these effects are cation-induced thylakoid membrane stacking (10, 12), increased PSII light utilization (21), increased PSII fluorescence yield (11, 21), and decreased PSII light utilization (21). Attempts to understand the mechanism of these effects have traditionally been made in terms of cation binding sites (2, 7, 25). However, in 1977, an alternative possibility was persuasively put forward by Barber et al. (4). They suggested that it would be more fruitful to think in terms of diffuse electrical layers at and very close to the thylakoid membrane surface to explain many cation effects. Subsequent analysis demonstrated the feasibility of this proposal (22-24), and the basic idea has recently been modified in an attempt to take into account the chemical diversity of different thylakoid regions (3).

Several years ago, we demonstrated that mild digestion of thylakoid membranes with the proteolytic enzyme trypsin could eliminate all of the above-mentioned cation effects (13, 14). Steinback et al. (25) subsequently verified these observations and showed that the main effect of trypsin was to digest a small fragment from the light-harvesting Chl a/b protein complex. These data seem to support the idea that cation effects are mediated by specific cation-binding sites, which should be located on or near the light-harvesting complex. However, the possibility that trypsin cleavage of the light-harvesting complex might lead to an increase in the negative charge on the thylakoid membrane surface by exposing previously hidden, negatively charged groups remained uninvestigated.

Here, we investigated this possibility and attempted to distinguish between the "specific sites" and "diffuse electrical layer" hypotheses for a number of cation effects.

MATERIALS AND METHODS

Chloroplasts were extracted from freshly harvested spinach (Spinacia oleracea) leaves in a 30 mM Tricine buffer (pH 8.0) also containing 10 mM NaCl and 0.4 mM sucrose and, where indicated, 3 mM MgCl₂. They were subsequently washed once and resuspended in the same buffer, unless otherwise stated. The reaction solution was the same medium minus sucrose, unless otherwise stated.

All fluorescence measurements were performed in a Perkin-Elmer MPF-3 spectrofluorimeter. 9-Aminoacridine fluorescence was excited at 398 nm with an intensity of about 1000 ergs cm⁻² s⁻¹ and measured at 456 nm. The chloroplast concentration was 20 µg Chl/ml, and the dye concentration was 25 µM. Under these conditions, the addition of 10 mM MgCl₂ increased the fluorescence yield, and no time-dependent quenching due to proton pumping was observed at the low exciting light intensity employed.

Chl fluorescence was excited at 440 nm and measured at 683 nm at a Chl concentration of 4 µg/ml, unless otherwise stated. Fluorescence induction was measured as previously described (15). The actinic light intensity was 4000 ergs cm⁻² s⁻¹ (Corning 4-96 filter) and the oscilloscope sweep time was 5 ms/division.

PSII activity was measured as O₂ evolution in the presence of the electron acceptor system 0.5 mM P-phenylenediamine 1 mM potassium ferricyanide. Illumination was with white light of about 6000 ergs cm⁻² s⁻¹. Doubling the light intensity led to an 80 to 90% increase in electron transport.

Fractionation of chloroplast membranes with digitonin was performed essentially as described by Boardman et al. (6). Prior to the fractionation step, the chloroplasts were washed with a solution containing 30 mM Tricine (pH 7.4), 10 mM NaCl, and also the appropriate concentration of MgCl₂. Chloroplasts then were suspended in the same medium at a Chl concentration of 300 µg/ml, and digitonin (2% w/v) was added to a final concentration of 0.5%. Treatment with digitonin was performed with agitation at 0 °C for 30 min when the chloroplast membrane-detergent mixture was diluted with the same buffer, maintaining unchanged the MgCl₂ concentration. Separation of the heavy and light membrane fractions was achieved by centrifugation at 10,000 g for 30 min. Chl was extracted with 80% acetone, and a/b ratios were determined according to the equations of Mackinney (18).

Electron microscopy was performed as previously described (8). Trypsin (type I) and soybean trypsin inhibitor (type I-S) were purchased from Sigma.

RESULTS

Searle et al. (24) have demonstrated that use of 9-aminoacridine as a probe for the electrostatic potential of the diffuse electrical layer associated with chloroplast membranes. The idea is that the monovalent cation, 9-aminoacridine, is attracted to the membrane surface by the negative potential. Within the electrical double

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layer, its fluorescence is quenched (probably by a mechanism of concentration quenching). Cation addition, by decreasing the negative surface potential, partially relieves this quenching. If trypsin treatment of chloroplast membranes were to change the surface-charge density and, hence, the negative potential of the diffuse electrical layer, this should be reflected by changes in 9-aminoacridine fluorescence.

Table I shows that mild digestion of chloroplast membranes with trypsin in the presence or absence of Mg ions leads to a small, but repeatable, decrease in 9-aminoacridine fluorescence. This trypsin treatment was sufficient to halve the Chl fluorescence yield in 4 min in the presence of 3 mM MgCl₂ and to eliminate completely the subsequent effect of 3 mM MgCl₂ when trypsinization was performed in the absence of Mg ions. When trypsin treatment was carried out in the presence of high Mg ion concentrations (50 mM), very small decreases in aminoacridine fluorescence were frequently observed which, however, were not statistically significant (unpublished data). It is not possible to quantitate experimentally increases of negative surface potential indicated by these fluorescence changes. However, on the basis of the theoretical considerations of Barber and Searle (5) and assuming that probe fluorescence responds similarly to equal changes in surface potential induced by monovalent and divalent cations, one can approximate that an increase in potential from -50 to -60 mV would lead to a decrease in dye fluorescence of about 0.6 units under our experimental conditions. Electrostatic potential of about -50 mV has been calculated for chloroplast membranes by Barber et al. (4). Thus, we tentatively conclude that trypsin treatment, sufficient to largely eliminate the stimulation of Chl fluorescence by 3 mM MgCl₂, is accompanied by an increase of approximately 20% of the negative surface potential. This value is intended to be understood as being orientative and indicative of the probable order of magnitude of the potential changes observed. These data do not, however, demonstrate that such changes are involved in the mechanism by which trypsin suppresses cation effects on chloroplast membranes.

The diffuse electrical layer theory would suggest that trypsin may counteract the effects of saturating concentrations of cations, increasing the negative membrane surface potential. In this circumstance, in order to reduce the electric potential to values previously attained by relatively low cation concentration, higher concentrations would be needed. We have titrated a number of cation-sensitive phenomena, which are influenced by trypsin digestion, before and after proteolytic treatment. These are: (a) cation-induced Chl fluorescence yield, (b) cation-induced membrane stacking, and (c) cation-induced increase in PSII photochemical efficiency.

Figure 1 represents an experiment in which the Chl fluorescence yield was titrated with different MgCl₂ concentrations before and after a brief treatment with trypsin. In the absence of proteolytic digestion, the concentration of MgCl₂ required to elicit half the maximal fluorescence response was around 0.6 mM and, after trypsin treatment, about 10 mM MgCl₂ was required. However, even at the highest Mg ion concentration, the reversal of the trypsin effect was not complete. It seemed possible that this may have been associated with the substantial decrease of fluorescence yield caused by trypsin in the absence of Mg ions. This suggests that trypsin treatment lowers the "basal" fluorescence yield (not due to added cations), as do experiments in which pronounced trypsin effects have been measured also in the presence of EDTA (unpublished data). Thus, if the data are replotted as the ratio of this basal fluorescence (Fig. 1B), the reversal of trypsin effects by high Mg ion concentrations is almost complete.

It is well known that cations increase Chl fluorescence largely by increasing the variable fluorescence (21). To check if the same applies upon addition of high Mg ion concentrations after trypsin digestion, we performed fluorescence induction studies. They show that high Mg²⁺ concentrations after trypsin treatment increase almost exclusively the variable fluorescence (Table II).

Figure 2 shows electron micrograph pictures of chloroplasts subjected to trypsinization sufficient to eliminate all membrane stacking, in the presence of 3 mM MgCl₂. Subsequent addition of high concentrations of MgCl₂ caused the reappearance of membrane appression. Although this stacking is not morphologically identical to that of chloroplasts not treated with trypsin, where clearly distinguishable grana can be seen, it frequently occurs in distinct zones in which a number of membranes seem to adhere together. To clarify whether or not these structures can be considered to be grana, we performed digitonin fractionation studies (Table III). Goodchild and Park (9) have shown that the

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**Table I. Effect of Trypsin Digestion of Chloroplast Membranes on Fluorescence Yield of 9-Aminoacridine**

Chloroplasts had been prepared with 3 mM MgCl₂ in all buffers. Washing and resuspension of the chloroplasts were performed with a medium containing 15 mM Tricine (pH 8.0), 5 mM NaCl, 3 mM MgCl₂, and 0.4 mM sucrose; trypsinization and fluorescence measurements were performed in the same medium minus sucrose in the presence or absence of 3 mM MgCl₂. Before adding trypsin (1 µg/ml), the chloroplasts were incubated at 20 µg Chl/ml for 3 to 4 min in the reaction buffer, containing 25 µM 9-aminoacridine. Data are arbitrary units of fluorescence.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fluorescence Yield after Time with Trypsin*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>None</td>
<td>23.9</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>31.0</td>
</tr>
</tbody>
</table>

* Variability of control and trypsin-treated samples as a function of time was estimated by linear regression. The difference between the regression coefficients of the two groups (control, + trypsin), assessed by analysis of variance (parallelism test), was significant at the 99% confidence level. Control values did not change during the experiment.

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**FIG. 1.** Titration of the Chl fluorescence yield with MgCl₂ before and after treatment of the thylakoid membranes with trypsin. Chloroplasts preparation and trypsin digestion were performed in the absence of MgCl₂. Proteolytic digestion was terminated by addition of a 50-fold excess of trypsin inhibitor, at which time the different concentrations of Mg²⁺ ions were added. The fluorescence yield was measured 4 min later. A, the unmodified fluorescence yields; B, the data from the same experiment are plotted as the ratio of fluorescence in the presence of Mg²⁺ to fluorescence measured in the absence of MgCl₂ (○), minus trypsin; (●), plus trypsin (2 µg/ml, 1 min). The Chl concentration was 20 µg/ml.
Table II. Effect of Different Concentrations of MgCl₂ on Variable and Nonvariable Components of Chl Fluorescence, before and after Treatment with Trypsin

Chloroplasts were prepared in the presence of 3 mM MgCl₂ and then treated (at 20 μg Chl/ml) with or without trypsin (Tryp; 1 μg/ml, 4 min) in the presence of 3 mM MgCl₂. Trypsin digestion was terminated upon addition of a 50-fold excess of trypsin inhibitor. Chloroplasts were subsequently washed in a solution containing 15 mM Tricine (pH 8.0), 5 mM NaCl, and 0.4 M sucrose and resuspended in the same buffer at different concentrations of MgCl₂. Measurements of fluorescence induction were carried out as described. Data are arbitrary units of fluorescence.

<table>
<thead>
<tr>
<th>Fluorescence Induction at Following Magnesium Concentrations</th>
<th>Fluo-</th>
<th>Parameter</th>
<th>Scence</th>
<th>0</th>
<th>5 mM</th>
<th>10 mM</th>
<th>50 mM</th>
<th>-Tryp</th>
<th>+Tryp</th>
<th>-Tryp</th>
<th>+Tryp</th>
<th>-Tryp</th>
<th>+Tryp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Tryp</td>
<td>34</td>
<td>17</td>
<td>111</td>
<td>20</td>
<td>103</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Tryp</td>
<td>21</td>
<td>17</td>
<td>28</td>
<td>20</td>
<td>38</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Table III. Effect of Various Concentrations of Mg²⁺ Ions on Fractionation of Chloroplast Membranes by Digitonin, before and after Treatment with Trypsin

For details of the experimental procedures, see Figure 2 and "Materials and Methods." Trypsin treatment was 1.2 μg/ml for 4.5 min. Ratio indicates the Chl a/b ratio and Yield indicates the percentage of the total Chl a + Chl b found in that particular fraction.

<table>
<thead>
<tr>
<th>Mg²⁺ Conc.</th>
<th>Light Fraction</th>
<th>Heavy Fraction</th>
<th>Light Fraction</th>
<th>Heavy Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio</td>
<td>Yield</td>
<td>Ratio</td>
<td>Yield</td>
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<tr>
<td>0</td>
<td>2.83</td>
<td>61</td>
<td>2.68</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>4.03</td>
<td>15</td>
<td>2.63</td>
<td>85</td>
</tr>
<tr>
<td>50</td>
<td>3.93</td>
<td>19</td>
<td>2.53</td>
<td>81</td>
</tr>
<tr>
<td>100</td>
<td>3.66</td>
<td>30</td>
<td>2.42</td>
<td>70</td>
</tr>
</tbody>
</table>

Table IV. Effect of Various Concentrations of MgCl₂ on Reduction of p-Phenylenediamine at Low Light Intensity, before and after Treatment with Trypsin

Trypsin treatment (70 s, 1.6 μg/ml) of chloroplasts (20 μg Chl/ml) was terminated by addition of a 50-fold excess of inhibitor. Units are nano electron equivalents/μg Chl-h. Incubation with MgCl₂ was for 4 min before commencing the measurement.

<p>| Effect of Following Salt Concentrations: |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>5 mM</th>
<th>10 mM</th>
<th>15 mM</th>
<th>25 mM</th>
<th>50 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Trypsin</td>
<td>48</td>
<td>72</td>
<td>68</td>
<td>70</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>+Trypsin</td>
<td>46</td>
<td>48</td>
<td>52</td>
<td>50</td>
<td>46</td>
<td>42</td>
</tr>
</tbody>
</table>

MgCl₂ concentrations after trypsin treatment are in fact grana. Table III shows that, although there is a clear relationship between the Mg²⁺-induced changes in Chl a/b ratio and yield in the light and heavy digitonin fractions in the absence of trypsin treatment, this is not the case after digestion with trypsin. In the latter case, Mg²⁺ exerts a strong influence on the yield of the light and heavy fractions while having no effect on the Chl a/b ratios. This aspect is treated in more detail elsewhere (17).

In Table IV, data are presented from an experiment in which the effect of Mg ions on PSII light utilization was determined at different MgCl₂ concentrations, with chloroplasts treated or not with trypsin. This treatment was sufficient to eliminate the well-known effect of Mg²⁺ on PSII light utilization. Increasing the concentration of MgCl₂ up to 50 mM after trypsin treatment did not reverse this effect of trypsin to the slightest degree.

DISCUSSION

Experiments reported here with 9-aminocaridine demonstrate that trypsin treatment of chloroplast membranes significantly increases the negative electrostatic potential at and near the membrane surface. The protolytic treatment required to elic it this effect is approximately equivalent to that required to eliminate the effects of saturating concentrations of Mg²⁺ ions on Chl fluorescence yield, membrane stacking, and PSII light utilization. Increasing the cation concentration after trypsin digestion is shown to be effective in substantially reversing these trypsin effects on both the Chl fluorescence yield and membrane stacking. This phenomenon is predicted by the diffuse electrical layer hypothesis (3, 4), where cations are expected to lower the negative surface potential by concentrating in a diffuse layer near the membrane surface. Thus, any increase in negative surface potential should be diminished by increasing the cation concentration in the medium.
We conclude that the diffuse electrical layer hypothesis satisfactorily explains the effects of trypsin in suppressing the effects of saturating concentrations of cations on Chl fluorescence yield and membrane stacking. Previous studies (1, 10, 14) have suggested that the cation-stimulated Chl fluorescence is associated with grana formation. The data presented here, which demonstrate a similar basic mechanism for both phenomena, support this conclusion.

Increasing the cation concentration after trypsin digestion does not increase PSII efficiency. Thus, it is clear that the trypsin suppression of this cation effect is not via the increase in electrostatic potential. It probably indicates that the basic cation interaction required to stimulate PSII is different from that described by the diffuse electrical layer hypothesis. It is possible that specific cation binding sites are involved. Such a conclusion is in complete accord with our earlier data, indicating that the effect of cations on PSII is able to be dissociated from the other effects on fluorescence and membrane stacking (13, 14, 16).

Recently both Mullet and Arntzen (20) and McDonnell and Staehelin (19) have demonstrated that, when the light-harvesting Chl-protein complex is incorporated into artificial membranes, cations can cause adhesion between these membrane surfaces. This "membrane stacking" is eliminated by trypsin treatment. These data are interpreted as demonstrating that this Chl-protein complex is the "stacking factor." It seems likely that the trypsin effects reported by these authors (19, 20) can be explained in terms of an increase in the negative surface potential of the liposome-Chl-protein complex membranes and that the cation-mediated stacking is controlled by the diffuse electrical layer associated with these membranes. The data of Mullet and Arntzen (20) and McDonnell and Staehelin (19) may demonstrate a molecular specificity at the level of the weak attractive forces required to hold the membranes together once the negative electrical field has been sufficiently masked by cations. However, this has not yet been definitively demonstrated and would require that other thylakoid membrane proteins when incorporated into artificial membranes do not mediate cation-induced stacking.

ADDENDUM

After this work had been submitted for publication, an article by Nakatani and Barber (Nakatani HY and Barber J 1980 Biochim Biophys Acta 591: 82–91) was published, in which they demonstrate, using the technique of particle electrophoresis, that trypsin treatment of chloroplast membranes probably increases the negative surface potential.

LITERATURE CITED