Effect of Trypsin Treatment of Photosystem I Particles on the Electron Donation to P700

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ABSTRACT

Proteolysis of photosystem I particles had no effect on P700 oxidation but did inhibit the rate of P700 reduction. The $V_{\text{max}}$ values were decreased for both dichlorophenol and plastocyanin, but the $K_m$ values were unaffected indicating that trypsin treatment altered electron transfer rather than the binding of the donor to the photosystem I complex. The salt dependence of P700 reduction was unaffected. The effects of P700 reduction were the same for the preparations of different workers (Shiozawa, Alberte, Thornber 1974 Arch Biochem Biophys 165: 388; and Bengis, Nelson 1975 J Biol Chem 250: 2783).

In both cases, the 70-kilodalton, chlorophyll-containing polypeptide was digested confirming its role in transferring electrons from plastocyanin to P700. The fact that the preparation of Shiozawa et al. lacks subunit (III) but still used plastocyanin as the electron donor rules out a role for this subunit as “the plastocyanin binding protein.” Subunit III was also digested in the Bengis and Nelson preparation.

Treatment with proteolytic enzymes has been used to study the structure and function of the plastoplast membrane. Experiments involving protease treatment of intact thylakoid membranes have shown that the pigment protein complexes were less susceptible to proteolytic attack than were the peripheral proteins (6, 17, 19, 24). However, extensive proteolysis did lead to breakdown of the pigment protein complexes (6, 14). In some cases, the fragmented polypeptides remained attached to the complex until the Chl molecules were removed (14, 16) indicating a structural role for the Chl molecules.

We have extended these studies to include the effects of trypsin treatment on isolated PSI particles in order to identify the functions of the individual polypeptides in the complex. We have used two types of PSI preparations in these studies. One is the preparation of Shiozawa et al. (23) which contains Cyt b6 and Cyt f in addition to a 70-kD polypeptide which contains Chl a and P700 (1). The second preparation is that of Bengis and Nelson (3, 4) which contains (in addition to the 70-kD subunit), the secondary electron acceptors and a 20-kD subunit (subunit III) which has been implicated in the binding of plastocyanin (11).

MATERIALS AND METHODS

Isolation of PSI Particles and Plastocyanin. PSI particles were isolated from spinach (Spinacia oleracea L.) according to the method of Shiozawa et al. (23) as modified by Gross and Grenier (10). Unless otherwise mentioned, most of the work was done using these particles. After elution of the particles with 300 mM phosphate buffer (pH 7.0) containing 0.05% Triton X-100, the particles were dialyzed against two changes for 12 h each of 0.05% (w/v) Triton X-100. PSI particles isolated according to this method had a Chl/P700 of 100 to 120 and Chl a/b of 7 to 9. For the purpose of comparison, PSI particles containing secondary electron transport components were isolated according to the method of Bengis and Nelson (3, 4) having a Chl/P700 of 80 and Chl a/b of approximately 8. These particles were also dialyzed against 0.05% Triton X-100 (w/v).

Plastocyanin was isolated by the procedure described by Davis and San Pietro (7) and its concentration was determined using an extinction coefficient of 4.9 nm$^{-1}$ cm$^{-1}$ at 597 nm as described by Davis and San Pietro (7).

Measurements of P700 Recovery (P700+ Reduction). The kinetics of P700 recovery were determined using an Aminco DW-2a spectrophotometer in its split beam mode of operation as described by Gross (9). The rate of P700+ reduction was calculated from the initial slope of the dark recovery. Actinic blue light from a 500-w slide projector was isolated using a Corning 4-96 and Bausch and Lomb 90-1-540 interference filter (I = 4.7 $\times$ 10$^4$ ergs cm$^{-2}$ s$^{-1}$). A Schott 695-nm highpass cutoff filter was used to protect the photomultiplier from the actinic light source. The measuring wavelength was 700 nm (resolution 3.0 nm). The P700 content of the sample was calculated using an extinction coefficient of 64 nm$^{-1}$ cm$^{-1}$ as described by Hiyama and Ke (12). $K_m$ and $V_{\text{max}}$ values were determined from the double-reciprocal plots by using a least-squares analysis (see Ref. 9).

Measurements of Fluorescence Spectra. Fluorescence emission and excitation spectra were recorded using a Spex Fluorolog Spectrofluorimeter. A Tracor Northern signal averager was used to store and analyze the fluorescence spectra.

Both emission and excitation spectra of Chl a in the PSI particles were measured at right angles to the excitation beam. For the measurement of emission spectra, the sample was excited at 435 nm (half bandwidth = 10 nm). Excitation spectra were recorded at 730 nm. Samples were suspended in 10 mM Tris-Cl (pH 8.2) at a Chl concentration of 1.0 and 10.0 µg/ml for the measurement of emission and excitation spectra, respectively.

The IMSL (International Mathematical and Statistical Libraries) were used to smooth fluorescence spectral data by cubic spline polynomials. Making use of the known statistical nature of the data (Poisson), the relative weights of the raw data and the standard deviation of the raw data, were used to central the degree of smoothing. In addition, all spectral data were corrected for tube sensitivity and background counts were subtracted. For the difference spectra, the two curves were normalized at their peak or at 680 nm. The computer and other facilities of The Ohio State University Instruction and Research Computer Center were used for all work.

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Trypsin Treatment. PSI particles (100 µg Chl) suspended in 0.05% Triton X-100 (w/v) were treated with 50 µg/ml trypsin (stock 1 mg/ml) (Chl:trypsin, 2:1 w/w) at room temperature. After the incubation of the particles with trypsin, 10 µl of 10 mM PMSF* (in n-propanol) were added for each 1 ml of incubation mixture, to inhibit the trypsin reaction. The control and trypsin treated particles were applied to a hydroxylapatite column which had been preequilibrated with 10 mM phosphate buffer (pH 7.0) and washed with 10 mM phosphate buffer (pH 7.0). PSI particles were then eluted with 300 mM phosphate buffer containing 0.05% Triton X-100 and dialyzed against 0.05% Triton X-100 (w/v). To prepare samples for electrophoresis, an aliquot of PSI particles treated with trypsin, followed by PMSF treatment for 10 min, was solubilized in the sample buffer containing SDS. Trypsin was added directly to the reaction cuvette at a Chl:trypsin ratio of 2:1, by weight, for the study of the time dependence of the inhibition of P700 recovery.

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis under denaturing conditions was performed according to the method of Kirchansi and Park (15) using 10% acrylamide slab or tube gels at constant current. SDS was added to the sample to give a final concentration of 2% (w/v). After the addition of SDS, the samples were incubated at room temperature for 30 min. Samples were not boiled before electrophoresis. The polypeptides were stained with Coomassie brilliant blue G-250 following the procedure of Holbrook and Leaver (13). Polypeptide bands were scanned using a Gilford spectrophotometer with a gel scanner.

The standard curve for the determination of the apparent mol wt was constructed using BSA (68,000), glutamate dehydrogenase (53,000), aldolase (40,000), chymotrypsinogen (25,700), RNase (13,700), and Cyt c (11,700).

Chl concentrations were determined using equations of Arnon (2).

RESULTS

Effect of Trypsin on the Kinetics of P700 Recovery. PSI particles show a reversible light-induced oxidation of P700 followed by a dark reduction of P700+. When the light was turned off, in the presence of 2 µM DCIP as the electron donor, the P700 recovered with a half-time (t1/2) of 4 s (Fig. 1A). The initial slope of the recovery of P700 in the dark (P700+ reduction) is, therefore, a measure of the steady-state rate of the electron transport through PSI.

Trypsin treatment of PSI particles increased the t1/2 of P700 recovery to 8 s. A calculation of the rate of P700+ reduction from the initial slope of the P700 recovery showed a 50% inhibition of the rate of P700+ reduction (Fig. 1B). Incubation of control PSI particles for 40 min at room temperature caused no further change in the rate of P700 recovery.

Trypsin treatment of PSI particles caused the inhibition of P700 recovery after short times of incubation (Fig. 2). The decrease in the rate of P700 recovery was fast initially, followed by a slower decrease. Incubation of PSI particles with trypsin for 2 min at room temperature caused a 40% decrease in the rate of P700+ reduction. Further incubation of PSI particles with trypsin for up to 40 min caused an additional 20% decrease in the rate of P700 recovery. In contrast to the effect on the P700 recovery, P700 oxidation measured at 430 nm using either 650 or 710 nm actinic light was not affected (not shown). These results suggest that the site of P700+ reduction is more exposed than the site of P700 oxidation.

The effect of varying the concentrations of trypsin is shown in

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* Abbreviations: PMSF, phenyl methane sulfonyl fluoride; DCIP, dichlorophenol indophenol.

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Fig. 1. Effect of trypsin treatment on kinetics of P700 recovery in isolated PSI particles. PSI particle (100 µg Chl ml⁻¹) isolated according to method of Shiozawa et al. (21) were treated with 50 µg of trypsin (Chl:trypsin = 2) for 10 min at room temperature. Kinetics of P700+ recovery were measured by suspending PSI particles (10 µg Chl/ml) in 10 mM Tris-Cl (pH 8.2) in presence of 0.7 mM ascorbate plus 2 mM DCIP to measure recovery kinetics. Other conditions are as described in "Materials and Methods."

Fig. 2. Effect of trypsin treatment of PSI particles on rate of P700 recovery, as a function of time of incubation. Rate of P700 recovery was calculated from initial slope of dark recovery. Other conditions are given in "Materials and Methods" and Figure 1.

Figure 3. Low concentrations of trypsin were effective in inhibiting the P700 recovery. A 50% inhibition in the rate of P700 recovery was obtained using 1.5 µg of trypsin/ml (Chl:trypsin = 6). Further increases in the trypsin concentration had no significant effect.

We determined the effect of trypsin treatment on the Kₐ and Vₘₐₓ values for natural and artificial electron donors (Table 1). The Vₘₐₓ value was decreased for both natural (plastocyanin) and artificial (DCIP) electron donors whereas the Kₐ values were unaffected. These results show that the electron transfer from the
were included for each determination from plots using cyanin titrations. Increasing concentrations of P700 were treated with increasing concentrations of trypsin for 10 min and kinetics of P700 recovery were measured as described in "Materials and Methods" and Figure 1.

Table 1. Effect of Trypsin Treatment on P700+ Reduction using DCIP and Plastocyanin as Electron Donors

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Electron Donor</th>
<th>Trypsin Treatment</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (( \mu M ) mg(^{-1}) Chl h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shiozawa et al. (21)</td>
<td>DCIP</td>
<td>-</td>
<td>17</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Shiozawa et al. (21)</td>
<td>Plastocyanin</td>
<td>-</td>
<td>13</td>
<td>462</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Bengis and Nelson (3, 4)</td>
<td>Plastocyanin</td>
<td>-</td>
<td>3.2</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.5</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

Donor to P700+, not the binding of the donor, is affected by trypsin. Both preparations of PSI particles showed similar abilities to use plastocyanin as an electron donor. Mg\(^{2+}\) ions were an absolute requirement in both cases (not shown). Moreover, the rate of P700+ reduction is equally sensitive to trypsin in both preparations.

FIG. 3. Effect of trypsin treatment of PSI particles on rate of P700 recovery as a function of trypsin concentration. PSI particles (10 \( \mu \)g Chl/ml) were treated with increasing concentrations of trypsin for 10 min and kinetics of P700 recovery were measured as described in "Materials and Methods" and Figure 1.

FIG. 4. Effect of ionic strength on inhibition of P700 recovery by trypsin. PSI particles (containing 10 \( \mu \)g Chl/ml) were incubated in 10 mM Tris-Cl (pH 8.2) and 2 mM Na-ascorbate and 5 \( \mu M \) plastocyanin various concentrations of MgCl\(_2\). Other conditions were as described for Figure 1. Slopes were determined using a least-squares analysis and were 29.6 ± 1.6 and 30.5 ± 1.6 for control and treated cases, respectively. Sixteen data points were used for each line.

FIG. 5. Fluorescence emission spectra of control (-----) and trypsin-treated (---) PSI particles. PSI particles were suspended in 10 mM Tris-Cl (pH 8.2) at 1 \( \mu \)g Chl/ml. The fluorescence was detected at right angle to excitation beam. Excitation wavelength, 436 nm; resolution, 1 nm.
Fig. 6. Fluorescence excitation spectra of control (— — —) and trypsin-treated (---) PSI particle at 730 nm. PSI particles were suspended in 10 mM Tris-Cl (pH 8.2) at 10 μg Chl/ml in 1 mm path length cuvette. Emission wavelength was set at 730 nm. Resolution of excitation beam was 1 nm. Difference spectrum (control-treated) is also shown (-----).

CONTROL
TRYPsin TREATED

Fig. 7. Effect of trypsin treatment on polypeptide composition of the PSI particles. PSI particles were isolated according to method of Shiozawa et al. (21) and treated with trypsin as described for Figure 1. SDS gels and densitometer scans were carried out as described in “Materials and Methods.”

Cations have been shown to increase the rate of P700+ recovery (5, 8, 9, 11, 17, 18) by shielding the net negative charge on the PSI particles (25). We examined the effect of trypsin treatment on the salt dependence of P700+ reduction in order to determine whether trypsin affected the net charge on the PSI particles. The results are plotted according to the Debye-Hückel formulation (25) (equation 1):  

$$\log v = \text{constant} + 1.018 Z_a Z_b (\text{IS})^{1/2}$$  

(1)  

The slope of a plot of log v versus (IS)−1/2 is proportional to the
net surface charge in this formulation as it is in the Guoy-Chapman formulation. Trypsin had no effect to within the limit of error (Fig. 4). Similar results were obtained using plastocyanin as the electron donor, and with the Bengis and Nelson preparation. Measurements of 9-aminoacridine fluorescence (22) also showed that trypsin had no effect on the net surface charge (not shown).

Trypsin treatment had no effect on the content of either Cyt f or Cyt b5 as measured by oxidized-reduced difference spectra (not shown).

**Effect of Trypsin Treatment on the Fluorescence Spectra.** PSI particles of Shiozawa et al. (23) show an emission peak at 682 nm at room temperature and a satellite band in the region of 720 to 740 nm (Fig. 5). This is in agreement with the reported spectrum of Shiozawa et al. (23). Trypsin treatment caused a change in the fluorescence spectrum in the red region. The peak of emission was shifted toward the blue on proteolysis and showed a slight decrease in the emission intensity. In the case of trypsin-treated particles, the fluorescence peak was observed at 680 nm. There was no significant change in the long wavelength part of the spectrum.

In order to find out whether trypsin treatment removes or alters the environment of the antenna Chl a molecules, we have compared the excitation spectrum of trypsin-treated particles with that of control particles (Fig. 6). The measuring wavelength was 730 nm and the resolution was 1.0 nm. A peak at 669.5 nm was observed in the excitation spectrum of untreated PSI particles. This suggests that the fluorescence at 682.5 nm originates from the Chl antenna absorbing around 669 nm. We do not find much indication of Chl678. It might be possible that extensive washing with Triton X-100 may remove long wavelength Chl forms. A prominent shoulder around 685 nm was also observed in the excitation spectrum. Proteolysis of PSI particles altered the Chl a excitation spectrum. In the case of trypsin-treated particles, the excitation peak was observed at 668 nm as compared with the peak at 669.5 nm for the untreated particles. In addition to the blue shift upon proteolysis in the excitation spectrum of PSI particles, the relative intensity around 685 nm was lower and the shape of the shoulder was altered. The difference spectra (untreated-treated excitation spectra) showed the presence of two positive peaks at 682 and 672 nm and a negative band around 668 nm. Although the accuracy of the measurements is such that the changes we observe are experimentally significant, we must conclude that the environment of the Chl molecules is not dramatically altered by trypsin treatment.

**Effect of Trypsin Treatment on the Polypeptide Composition of the PSI Particles.** PSI particles isolated according to the method of Shiozawa et al. (23) contained the following peptides: 71, 66, 63, 43, 37, and 30 kD (Fig. 7). The band at 70 kD has been shown to contain the Chl and P700 (1). This band shows anomalous behavior in this gel system and may appear as multiple bands migrating between 60 and 70 kD. In addition to the 71-kD band, we observed additional bands and 47 and 30 kD which correspond to Cyt b5 and Cyt f (26), respectively, which are known constituents of this preparation. We also observed contamination by coupling factor at 53 and 37 and possibly at 60 kD (20). In some preparations, the contamination by coupling factor was much more prominent.

Treatment by trypsin caused a decrease in the 71-kD band with the appearance of bands 53, 13.5, and 10.5 kD. Thus, it appears that trypsin removed a small piece from the 71-kD band. Because the 70-kD band is the only one affected, it implies that it is involved in electron donation to P700⁺ using both plastocyanin and artificial electron donors. On the other hand, the fact that there was only a slight alteration in this band is consistent with the observation that neither the total P700 signal nor its rate of oxidation were affected by trypsin treatment. It is also consistent with the observation that there were only minor changes in the environment of the Chl molecules. As expected from the difference spectra, there was no change in the polypeptides corresponding to Cyt f and Cyt b5.

We obtained the same results for the Bengis and Nelson (3, 4) preparation with one exception (Fig. 8). In the control gels, we observed bands at 88, 69, 60, 56, 47, 40, 32, 26.5, 23, 20, 15, and 9 kD. As discussed above, 60- and 69-kD bands are different forms of the 70-kD Chl a-containing subunit (1). The band migrating at 88 kD (which is also occasionally seen in the Shiozawa et al. [23] preparation) probably corresponds to the 95-kD Chl protein observed by Wessels and Borchert (27).

The 26-, 20-, and 15-kD polypeptides are can be identified as bands II, III, and V of the Bengis and Nelson preparation (3). Inasmuch as these three bands are missing from the Shiozawa et al. (23) preparation (Fig. 7), and both preparations carry out electron transport from plastocyanin (or an artificial electron donor) to O₂, they cannot be involved in the oxidizing side of PSI. In particular, band III, cannot be a 'plastocyanin-binding' protein because it is not required in order to use plastocyanin as an electron donor. Likewise, it cannot be a Mg²⁺-sparing protein since both preparations have the same Mg²⁺ ion requirements. These results do not rule out a role for these peptides on the reducing side of PSI between the primary acceptor and NADP⁺.

We do observe some contamination in our Bengis and Nelson preparation. We have a small amount of coupling factor (at 56 kD), some Cyt f (at 32 kD), and some polypeptides from the light-harvesting Chl a/b protein (at 23 kD).

Trypsin treatment cleaved the 60- and 69-kD bands causing them to migrate at 56 and 51 kD. Small peptides also appeared at 16 and 12 kD. Thus, as in the case of the Shiozawa et al. (23) preparation, trypsin cleaved a small part of the '70-kD' polypep-
tide. Since the effects on activity are the same for both preparations and the same peptide is cleaved in both instances, this provides further evidence for the participation of the 70-kD polypeptide in electron donation to P700'. It is of interest to note that the 20-kD polypeptide is also cleaved but it cannot be an obligate participant in electron donation to P700 since it is not present in the Shiozawa et al. (23) preparation.

DISCUSSION AND CONCLUSIONS

We have studied the effect of trypsin treatment on the activity and polypeptide composition of PSI particles isolated according to two different methods. Our aim was to identify the polypeptides of the PSI susceptible to trypsin attack and their role in the activity of the PSI particles.

On proteolysis, the PSI particles prepared according to the method of either Shiozawa et al. (23) or Bengis and Nelson (3, 4) showed a decrease in the rate of P700<sup>+</sup> recovery using both natural and artificial electron donors. In contrast, no effect was observed on the rate of P700 oxidation. These results suggest that the site of P700 oxidation may be buried inside the complex, and thus, inaccessible to proteolytic digestion.

When we determined the effect of proteolysis on the polypeptide composition of PSI, we found that the 70-kD polypeptide was cleaved in both preparations. This polypeptide has been shown to contain both Chl and P700 (1). Therefore, it is reasonable that cleavage of this peptide should affect both the donation of electrons to P700<sup>+</sup>. The relatively small effect on Chl<sub>a</sub> fluorescence suggests that the Chl molecules are not on the part of the 70-kD polypeptide that is cleaved by trypsin.

Bengis and Nelson (3, 4) and Haehnel et al. (11) have postulated that a 20-kD polypeptide (subunit III of the Bengis and Nelson preparation) is required for plastocyanin donation to P700<sup>+</sup>. Our results disagree with these observations. We find that the preparation of Shiozawa et al. (23) lacks subunit III but is still able to use plastocyanin as an electron donor. Moreover, they have the same Mg<sup>2+</sup> ion requirements. It is interesting to note that subunit III is cleaved on trypsin treatment in the Bengis and Nelson preparation, but has no apparent effect on P700<sup>+</sup> reduction beyond that observed when only the 70kD polypeptide was cleaved.

Our results on trypsin treatment of isolated PSI particles agrees with the results of Nelson and Notsani (21) for the effects of trypsin treatment on PSI in the chloroplast membrane. They also found that both the 70-kD subunit (subunit I) and the 20-kD subunit (III) were cleaved with the appearance of bands at 34, 26, and 10 kD.

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LITERATURE CITED