Trypsin-Mediated Removal of Herbicide Binding Sites within the Photosystem II Complex *

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Trypsin treatment of isolated chloroplast thylakoids resulted in a step-wise modification of surface exposed membrane polypeptides. Early effects of the protease action resulted in a decrease in inhibitory activity of atrazine, diuron, pyrazon, and bromacil, but an initial increase in the activity of bromnitrothymol and dinoseb. Direct measurements of atrazine binding demonstrated that decreased inhibitory activity corresponded to a decreased binding affinity in the treated membranes. Longer term effects of trypsin caused removal of atrazine binding sites and a concomitant block of electron transport chains. The data are consistent with a concept that the traizine receptor protein is a component of the electron transport chain which is successively degraded in two or more steps by protease attack.

Polyacrylamide gel electrophoresis of trypsin-treated membranes and sub-membrane fragments derived from these membranes revealed that several polypeptides are membrane surface exposed. The involvement of a 32000 dalton polypeptide in creating the atrazine binding site is discussed.

Introduction

The proteolytic enzyme trypsin can be used to selectively modify surface-exposed polypeptides in chloroplast membranes [1]. Over the last several years this procedure has been extensively developed to analyze components of the photosystem II (PS II) complex and to study the mechanism(s) by which photosynthetic herbicides such as diuron (DCMU) block PS II-mediated electron transport (see recent reviews in refs. [2–4]). We can summarize current information as indicating that the reaction center of PS II is relatively stable against trypsin attack, but one or more electron carriers acting on the reducing side of PS II are surface exposed and are modified in a time-dependent fashion after addition of trypsin to isolated chloroplast membranes. In spite of extensive studies by a number of laboratories, certain inconsistencies and controversies concerning the site of trypsin attack remain in the literature. It was initially implied that trypsin-treatment of isolated thylakoids results in herbicide-insensitive electron transport through the normal electron transport chain [5]. More recently, however, many investigators have concluded that herbicide-insensitive electron flow results from creation of a new, artificial side path for electron transfer to added electron acceptors, probably arising directly at the level of Q (the primary PS II electron acceptor) [3, 4, 6]). It has not yet been clarified whether removal of herbicide binding sites in the chloroplast membrane corresponds directly to modification of the apoprotein of an electron carrier acting in the electron transport chain, or whether the protein digested by protease attack is a "shield protein" which covers the PS II complex but may not actually be an electron carrier. Support for the latter hypothesis has come from recent studies by Croze *et al.,* [7] who found that two polypeptides can be selectively modified by trypsin in isolated PS II particles without blocking electron transport in these preparations.

It was a goal of the current study to conduct a detailed time-course of trypsin modification of chloroplast membranes and to analyze the resultant sam-

ples for the presence of herbicide binding sites, the binding affinity of these sites, and the ability of the samples to conduct non-cyclic electron transport through the intersystem portion of the chain. In addition, we have attempted to determine whether a specific polypeptide could be identified as the target site for trypsin attack within the PS II complex.

Materials and Methods

Chloroplast isolation

Forty grams of washed pea or spinach leaf tissue were homogenized in a blendor for 10 seconds in 100 ml of solution (4 °C) containing 50 mM Tricine-NaOH (pH 7.8), 10 mM NaCl and 0.4 M sorbitol. The homogenate was passed through 2 and then 8 layers of cheesecloth and the filtrate was centrifuged at $1000 \times g$ for 10 min. The chloroplasts obtained from the pellet were osmotically shocked by suspension in 40 ml of 10 mM Tricine-NaOH (pH 7.8), 10 mM NaCl. Broken, stroma-free thylakoids were then collected by centrifugation at $1000 \times g$ for 10 min and resuspended to a final Chl concentration 1–2 mg of Chl/ml in PSNM buffer (50 mM sodium phosphate (pH 7.2), 100 mM sorbitol, 10 mM NaCl and 5 mM $MgCl_2$).

Trypsin incubation

Trypsin incubations were carried out at room temperature in dim light. Membranes were brought to a final concentration of 100 µg Chl/ml with room temperature (21 °C) PSNM buffer Trypsin (TPCK, Millipore Corp., 223 enzyme units/mg protein) was added to a final concentration of 2 µg/ml (or as specified in text) and at various time intervals, 10–20 ml aliquots were removed to chilled centrifuge tubes containing 20 fold excess (40–80 µg) trypsin inhibitor (Soybean, Type 1-S, Sigma Chemical Co.). Membranes were pelleted at $4000 \times g$ for 5 min, and were washed once with cold PSNM buffer. Following final centrifugation, membranes were resuspended to 1 mg Chl/ml and kept on ice for subsequent assays.

Photosynthetic reactions

All photosynthetic reaction assays were conducted on isolated spinach thylakoid membranes in PSNM buffer, with 10^{-7} M gramicidin-D and 1 mM Nh_4Cl included as uncouplers of electron transport. Elec-

tron donors and acceptors were added as indicated in the text or figure legends. Photosynthetic inhibitors were prepared as concentrated stock solutions in absolute methanol. Addition volumes to assay media were in all cases less than 0.5% of the final volume.

Measurements of DCPIP photoreduction were carried out using a Hitachi Model 100–60 Spectrophotometer modified for cross-illumination as previously described [8]. Oxygen evolution was assayed with a Clark-type oxygen electrode (YSI, Yellow Springs Ohio). Whole chain electron transport to methyl viologen was monitored as O_2 uptake.

Herbicide binding assays

Trypsin-treated, washed chloroplast thylakoids were suspended in PSNM buffer. 50 µg Chl in a 1 ml volume were incubated for 3 min with ^{14}C-labeled atrazine (specific activity: 27.2 µCi/mg; added in 2–15 µl volumes to each assay tube). Chloroplasts were pelleted and an aliquot of the supernatant was removed for determination of the amount of unbound atrazine. Details of this procedure have been previously described [9–11].

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12–20% (w/v) linear polyacrylamide gradient slab gels [12] with a 1 cm 5% stacking gel incorporating the buffer systems of Laemmli [13]. Samples were prepared for electrophoresis and gels were fixed and stained for protein as previously described [1].

Detergent incubation and sucrose density centrifugation

Membrane fractionation using the detergent Triton X-100 and purification of membrane complexes on sucrose density gradients are described elsewhere [14]. For analysis of trypsin-treated membranes (subsequent to addition of trypsin inhibitor), thylakoids were washed 2 times unbuffered 100 mM sorbitol, 5 mM EDTA (pH 7.8), to remove bound Mg^{2+}. The membranes were then washed in 100 mM sorbitol and were resuspended to 0.5 mg Chl/ml in distilled H_2O. Membranes were incubated with 0.45% (w/v) Triton X-100 at room temperature in the dark for 30 min with gentle stirring. Centrifugation at $40000 \times g$ for 30 min, 4 °C following incubation removed undi-

gested membrane fragments from the preparation. The $40\,000 \times g$ supernatant was loaded onto 0.1–0.7 M sucrose gradients (containing 0.02% Triton X-100) which were cast above a 2 M sucrose cushion (5 ml). The gradients were centrifuged 16 hrs at $100\,000 \times g$ in a Beckmann SW-27 type swinging-bucket rotor. Fractions were subsequently collected from the gradients.

Results and Discussion

A. Trypsin affects on photosynthetic partial reactions

Trypsin-treated thylakoid samples were analyzed for electron transport using water as the electron donor in the presence of various artificial electron acceptors (Fig. 1). A parallel time course for loss of photochemical activity was observed when DCPIP, DAD, or MV were used as electron acceptors; approximately 50% of control activity was lost within 10 min of trypsin addition. In contrast, Hill reaction activity with potassium ferricyanide (FeCN) was affected during the first 10 min of trypsin incubation but then remained stable over the subsequent treatment periods. Electron flow using silicomolybdate
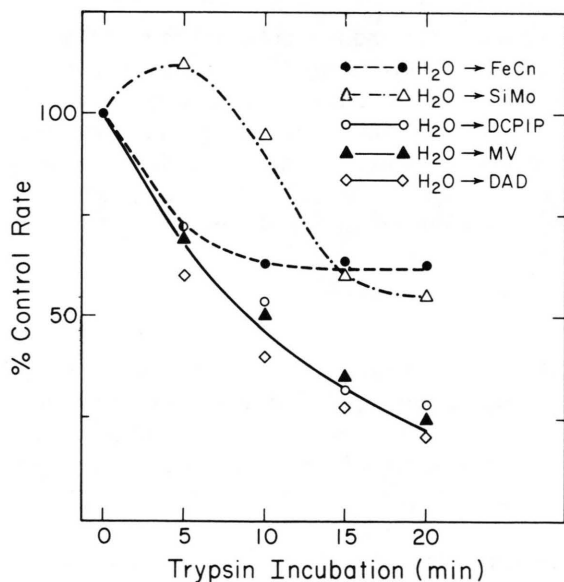

Fig. 1. Effect of trypsin on electron transport. Spinach thylakoid membranes (100 µg Chl/ml) were incubated with trypsin (2 µg/ml) for 0 to 20 min. Trypsin was inhibited by addition of ten fold excess of trypsin inhibitor. Membranes were washed once with PSNM buffer and assayed for electron transport activity as described in Methods. Rates are expressed as percent of control rate (no trypsin treatment).
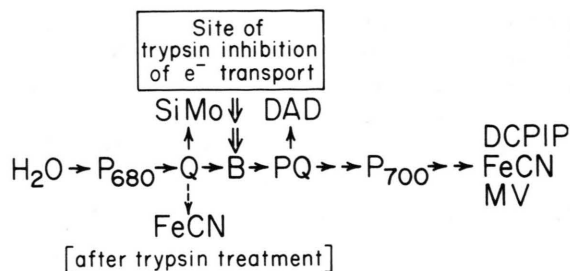

Fig. 2. Schematic summary of electron acceptor sites in the electron transport chain before and after trypsin treatment.

(SiMo) as the acceptor was initially stimulated (during the first 5 min treatment period), and then inhibited to approximately 50% of the control activity. Our interpretation of these data is summarized in Fig. 2. Acceptors which remove electrons from the chain at or after the level of plastoquinone (DAD, DCPIP, MV, and FeCN) suffer inhibition due to the inactivation of an electron carrier acting after Q. Since transfer of electrons to SiMo was not initially inhibited, the components acting up to the level of Q must be protected from trypsin attack. The carrier which is highly sensitive to trypsin treatments is most probably the protein-plastoquinone acting as the secondary PS II electron acceptor (B). This conclusion is in agreement with other recent studies [3, 4]. The initial stimulation of SiMo photoreduction by trypsin treatment is consistent with the fact that SiMo accepts electrons at the level of Q. Enhanced accessibility of SiMo to Q could be achieved by removal of surface-exposed regions of adjacent polypeptides. Long-term inhibition of SiMo reduction (10–20 min of trypsin treatment) is probably due to alterations in the structural organization of the PS II reaction-center and/or modification in the function of components on the oxidizing side of PS II.

Fig. 3 presents electron transport data for control thylakoids and membranes treated with trypsin for 15 min; the two samples were subjected to a range of atrazine concentrations. In control or treated membranes, 5 µM atrazine gave complete inhibition of non-cyclic electron transfer (water as an electron donor and MV as acceptor). It is to be emphasized that no herbicide-insensitive electron transport could be detected. Virtually identical data was obtained when diuron was substituted for atrazine in these experiments – that is, diuron could totally block non-cyclic electron transport. In a parallel set of experiments,
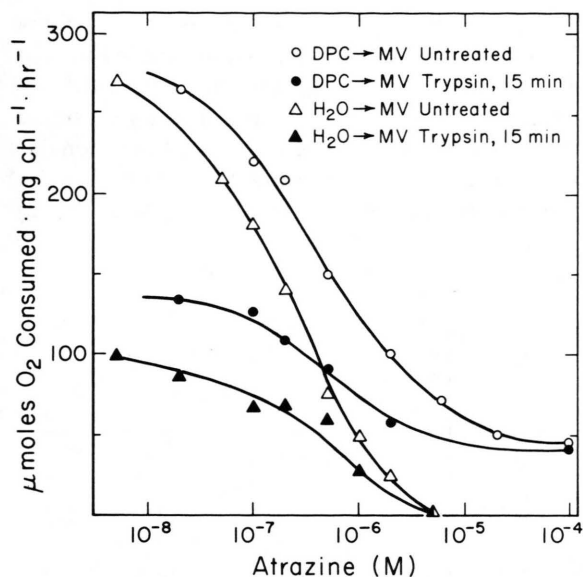
Fig. 3. Effect of the artificial electron donor, DPC, on electron transport (methyl viologen as electron acceptor) in control membranes and thylakoid membranes which had been treated with 2 µg/ml trypsin for 15 min.

optimize the rate of electron flow in membranes treated with trypsin for 15 min. At high FeCN concentrations (> 5 mM) diuron was nearly inactive in blocking FeCN reduction in trypsin-treated membranes. As was shown in Fig. 2, we interpret these data to indicate that a new site for FeCN reduction is exposed within the PS II complex after trypsin alteration of surface-exposed polypeptides of this complex. This interpretation is in agreement with conclusions of Renger and co-workers (4, 6).

When rates of electron transport from water to MV were measured in control and trypsin-treated chloroplast membranes (Fig. 3), it was observed that higher concentrations of atrazine were required to give 50% inhibition in the protease modified membranes. To more extensively characterize this phenomenon, Hill reactions using DCPIP as the electron acceptor were conducted using thylakoids treated with trypsin for 15 min. The data, presented in Fig. 4, demonstrate that there was a shift in the atrazine response curve. It should be noted that the data in

we have included DPC as an electron donor (Fig. 3). In untreated membranes, approximately 15% of the control rate of electron transport was insensitive to 100 µM atrazine. In trypsin-treated membranes, the rate of electron transfer from DPC to MV was nearly identical to untreated membranes at 100 µM atrazine; it should be noted that this represents approximately 30% of the control rate of electron flow in the trypsin-treated membranes whereas the absolute rate of electron flow is no different from that of the controls. This indicates that herbicide-insensitive electron transport is always detectable in chloroplast assays utilizing electron donors such as DPC; the trypsin treatment of chloroplast membranes does not create a PS II-mediated electron transport pathway involving intersystems electron carriers which are insensitive to herbicides. It is likely that the DPC dependent, herbicide-insensitive reduction of MV (or DCPIP) is PS I mediated and is due to DPC electron donation into the intersystem chain. In other experiments (data not presented) we have examined FeCN reduction in the presence and absence of atrazine or diuron before and after trypsin treatment. In control membranes, 1 mM FeCN was a saturating level of electron acceptor whereas > 5 mM was required to

Fig. 4. Effect of trypsin on the relative affinity of atrazine for its binding site as measured by inhibition of electron transport. Membranes (100 µg Chl/ml) were incubated with 2 µg/ml trypsin for 15 min. Ten-fold excess trypsin inhibitor was added to stop trypsin action. Membranes were washed once with PSNM buffer and assayed for electron transport activity in the presence of increasing concentrations of atrazine. Rates are expressed as the percent of control rate (no trypsin treatment). The control rate of DCPIP photoreduction in this experiment was 480 µ equivalents. mg chl^{-1} · h^{-1}.

this figure are presented as percent of control activity. I_{50} concentrations determined from these studies were compared to similar studies using diuron and bromnitrothymol (data not presented). The degree of decrease of atrazine affectiveness in trypsin-modified membranes was expressed by the ratio of the I_{50} concentration after treatment relative to the I_{50} concentration of control samples. It was found that the decrease in atrazine activity caused by trypsin treatment was significantly more than that observed for diuron and bromnitrothymol at similar time points; we emphasize that these inhibition data were obtained using the same stock suspensions of both control and trypsin-treated chloroplasts. The sug-
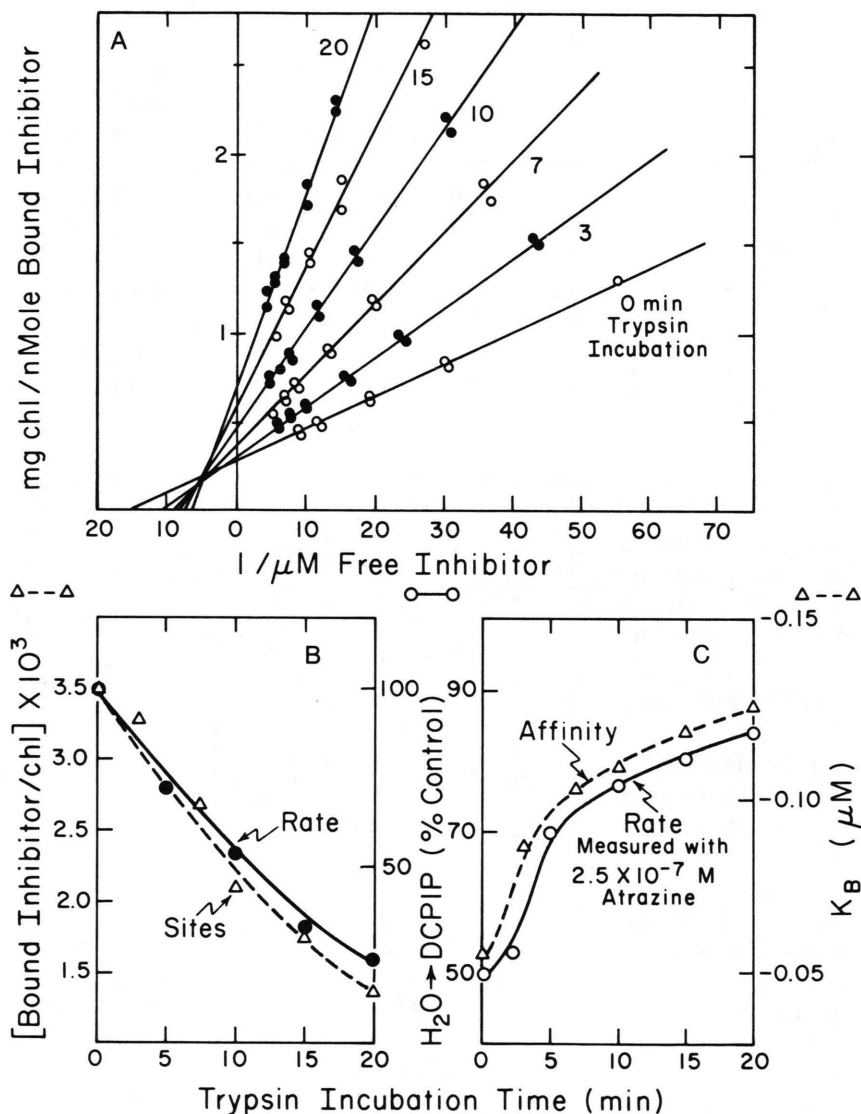

Fig. 5. A. ^{14}C-atrazine binding for thylakoid membranes incubated with 2 µg/ml trypsin for various times (0 to 20 min). Data, plotted in double reciprocal form, demonstrate both changes in atrazine affinity for the binding site (abscissa intercept) and loss of binding sites (ordinate intercept) over the trypsin time course studied. B. Comparison of data derived from Fig. 5 A demonstrating loss of binding sites, with that obtained from direct analysis of electron transport capacity (H_2O to DCPIP) at high light in uninhibited samples. C. Comparison of data derived from Fig. 5 A demonstrating loss of herbicide affinity with that obtained as described in Fig. 6, demonstrating change of affinity in electron transport assays with increasing time of trypsin incubation. Electron transport (H_2O to DCPIP) was measured in the presence of 2.5×10^{-7} M atrazine.

gestion from the data was that trypsin-treatment of thylakoids selectively alters the binding affinity of PS II for these herbicides.

B. Analysis of herbicide binding to trypsin-treated thylakoids

For the data in Fig. 5, a series of chloroplast samples were treated with trypsin for time periods ranging from 0−20 min. Each sample was subsequently analyzed for binding of radioactive atrazine. The data, presented in double-reciprocal form, allow an evaluation of the herbicide binding constant (abscissa intercept) and the number of herbicide binding sites on a Chl basis (ordinate intercept). It can be observed that initial periods of trypsin treatment resulted in a rapid change of binding affinity for atrazine, but only a limited change in the number of binding sites. Subsequently, there were only minor changes in the binding constant but a gradual loss of herbicide binding sites.

In Fig. 5 B, the measured number of herbicide binding sites in trypsin-treated chloroplast thylakoids is compared to the absolute rate of uninhibited, non-cyclic electron transport ($H_2O \rightarrow DCPIP$). The data clearly indicate that there is a time-dependent removal of herbicide binding sites by trypsin. The loss of binding sites paralleled the loss of non-cyclic electron transport. We conclude from these studies that the presence of the herbicide binding protein is essential for the function of components on the reducing side of PS II. This is consistent with, but does not prove, that the herbicide receptor protein is itself a component of the electron transport chain.

The affects of trypsin on herbicide affinity are compared to the activity of atrazine in blocking electron flow in Fig. 5 C. For this experiment, samples were treated with trypsin for 0−20 min. Aliquots removed from the incubation medium were tested for electron transport in the presence and absence of 0.25 μM atrazine; this herbicide concentration gave 50% inhibition of electron transport in the control samples. As is clearly evident, within 5 min there was a marked decrease in electron transport sensitivity to the herbicide. In parallel, from measurements of actual herbicide binding using radioactive atrazine, there was a decrease in the affinity of atrazine for the chloroplast membrane. The parallel time course

in change of atrazine affinity and inhibitory activity demonstrate that trypsin selectively alters a component of PS II in functionally active chains such that atrazine activity in these chains is diminished due to decreased binding affinity.

The fact that mild trypsin-treatment of chloroplast membranes selectively alters the affinity for atrazine lead us to examine the treated membranes for similar effects using other chemical families of PS II-directed herbicides. For these experiments it was necessary to use only electron-transport assays since radiolabeled herbicides were not available. The data, presented in Fig. 6, show that trypsin-treatment of chloroplasts results in a marked time-dependent decrease in diuron, pyrazon, and bromacil activity. In contrast, dinoseb and bromnitrothymol (phenol-type herbicides) increased in herbicidal activity in early
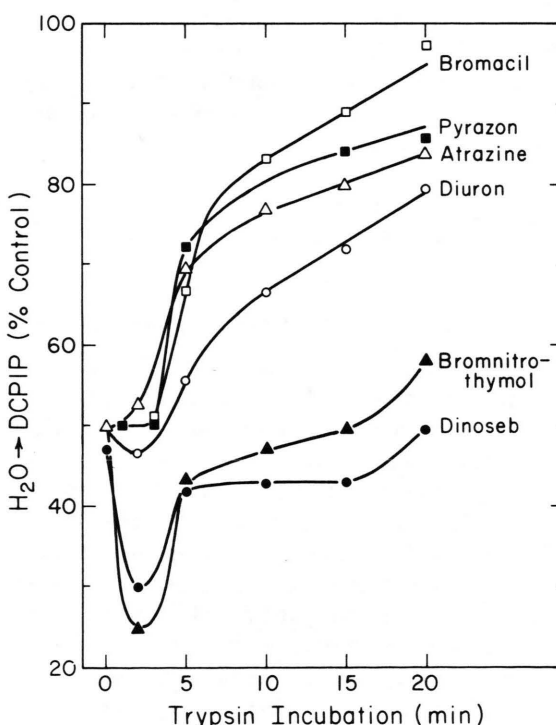

Fig. 6. Alteration of herbicide affinity following trypsin treatment. The I_{50} concentration (herbicide concentration required to inhibit control rates of PS II electron transport, by 50%) was determined for control (untreated) membranes for each herbicide used. The inhibition capacities of these I_{50} concentrations of bromacil, pyrazon, atrazine, diuron, bromnitrothymol and dinoseb were tested after 2 to 20 min trypsin treatment of thylakoid membranes. Results are presented as percent of control electron transport activity (H_2O to DCPIP) following each trypsin time point.

time periods of trypsin-treatment. This increased in activity was reversed over the 5–20 min treatment period. These changes in herbicide activity (relative measurement of affinity) are not related to gross structural changes of the membranes following trypsin treatment. We have earlier demonstrated that mild trypsin-treatment of chloroplast membranes results in an unstacking of the thylakoids and a dispersal of the large freeze-fracture particles within the lipid phase of the chloroplast membrane [1]. Similar structural alterations can be induced simply by suspending isolated, control chloroplasts in "low-salt" buffers [16]. We have monitored diuron and bromnitrothymol activities in "high-salt" (stacked) and "low-salt" (unstacked) membranes – there is no difference in inhibitory activity in these membranes (data not presented). We therefore conclude that the alterations in herbicide activity reported in Fig. 6 are due to an actual change in the microenvironment surrounding the herbicide binding site.

It should be noted that Boger and Kunert [17] have also reported differential changes in herbicide activity in trypsin-treated chloroplast membranes. They interpreted these data as indicating the various classes of herbicides are affected by different binding properties in the region of inhibitor action. Our data are consistent with their hypothesis.

C. Polypeptide alterations in trypsin-treated thylakoids

An ultimate goal of the biochemical analysis of herbicide affects on chloroplast membranes is to identify the specific membrane constituent which is the herbicide receptor. Since trypsin modification of the membrane results in step-wise loss of herbicide binding affinity and binding sites, it is likely that the herbicide receptor is a polypeptide. In an attempt to identify this polypeptide, we have used SDS-PAGE for analysis of trypsin-treated membranes. These experiments have been conducted with both stroma-free spinach and pea thylakoids. The latter have been used because we have previously obtained a more detailed identification of pea membrane polypeptides [1, 14, 18, 19]. The effect of incubation at two trypsin concentrations on isolated pea thylakoids is shown in the polyacrylamide gel of Fig. 7. More than 30 polypeptides can be resolved in the untreated chloroplast membranes (lane A of Fig. 7). Following trypsin treatment at 2 µg/ml for 15 min (lane B of Fig. 7), the most obvious change in mem-

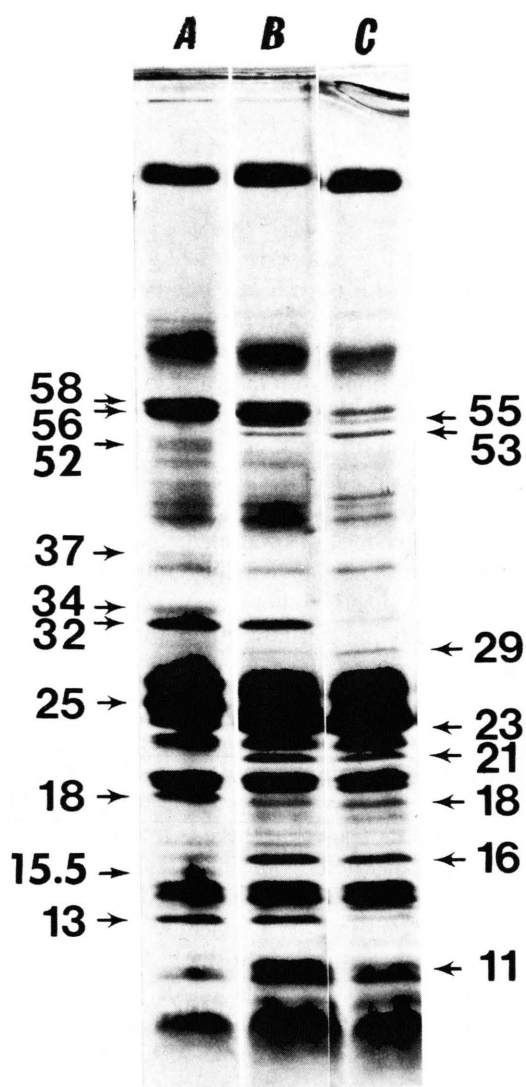

Fig. 7. Stained (coomassie blue) polyacrylamide gel of pea chloroplast thylakoids: A) untreated, B) treated with 2 µg/ml trypsin for 15 min, and C) treated with 20 µg/ml trypsin for 15 min. Following trypsin treatment and addition of trypsin inhibitor, samples were washed once with PSNM buffer prior to sample preparation for electrophoresis. SDS-PAGE was carried out as described in Methods.

brane polypeptide content were deletions of polypeptides at 52, 37, 34, 25, 18, and 15.5 Kdaltons. This corresponds to a time point at which there were major changes in atrazine affinity, and at which approximately 50% of atrazine binding sites were lost. At 20 µg trypsin/ml for 15 min (lane C of Fig. 7), approximately fourteen polypeptides had changed in apparent molecular weight, although the cataloging
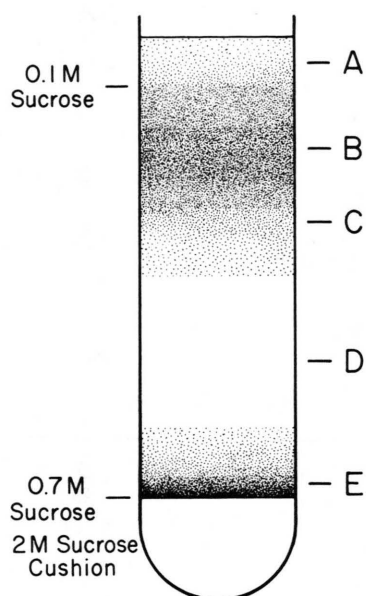
Fig. 8. Schematic diagram of sucrose density gradient following 16 h centrifugation at 100 000×*g* of "low-salt"membranes treated with Triton X-100. Shading represents relative density of chlorophyll throughout the gradient. Localization of various protein or Chl protein complexes are indicated: The various regions of the gradient labeled A−E represent relative enrichments of: A) proteins solubilized (released from membrane) by Triton X-100; B) proteins of the LHC; C) proteins of the PS II complex; D) proteins of the chloroplast coupling factor; E) proteins of the PS I complex.

of these changes was made difficult by the fact that new staining bands appeared due to partial proteolytic digestion of various proteins. These membranes were inactice in non-cyclic electron transport from H_2O to MV. Because the analysis of entire membrane samples is complex due to the diversity and multiplicity of protein changes, we have further characterized the trypsin-treated membranes by fractionation on sucrose density gradients following detergent solubilization.

Fig. 8 diagramatically represents the distribution of chlorophyll over a sucrose gradient containing thylakoid sub-membrane protein complexes; we have previously reported the localization of various functional complexes within these preparations [14]. Fractions A−E are preferentially enriched in: A = solubilized proteins released from the membranes; B = light-harvesting chl a/b complex (LHC); C = PS II; D = coupling factor; E = PS I.

A polypeptide migrating in polyacrylamide gels with an apparent molecular weight of 11 Kd was detected in gradient zone A after all trypsin treatments

(Fig. 9 A). Plastocyanin can be purified from this sample and runs as a homogeneous band of 11 Kd. We therefore conclude that either 2 or 20 μg trypsin/ml did not disrupt the thylakoids over 15 min and the internally localized, soluble plastocyanin is not digested by the protease. All subsequently discussed changes therefore refer to surface-exposed proteins.

Zone B (Fig. 8) of the sucrose gradient was enriched in Chls a and b and polypeptides of 23−28 Kd (Fig. 9 B); these have been shown to be components of the light-harvesting pigment-protein complex (LHC) [19]. Trypsin treatment resulted in the cleavage of surface-exposed segments of these proteins to a 23 Kd species [1]. Based upon studies of membranes deficient in the LHC [20, 21], which still retain herbicide sensitivity, we conclude that the LHC protein modification is not involved in the altered herbicide binding sites.

Zone C (Fig. 9 C) of the gradient contained polypeptides of the LHC as well as several polypeptides associated with photosystem II [14]. Mild trypsin treatment altered the mobility of a 52 Kd polypeptide, removed a 34 Kd component, and decreased the staining intensity of a 32 Kd polypeptide. Higher trypsin levels totally removed the 32 Kd component and further altered polypeptides in the 42−44 Kd size classes. These polypeptides are all components of purified PS II preparations [7].

Zone D (Fig. 9 D) of the sucrose gradient was enriched in components of the coupling factor. The α, β, and γ, subunits, resolved in this gel system (58, 56, 37 Kd respectively), were extensively modified by 2 μg trypsin/ml and were totally degraded at the higher trypsin levels. Other polypeptides of the $CF_1−CF_0$ complex did not stain intensely in our gels and can not be visualized.

Zone E (Fig. 9 E) of the sucrose gradients was highly purified PS I; characteristics of these preparations have been described [18, 22]. The pigmented reaction-center complex (labeled CPI) and the apoproteins of the CPI (68, 66 Kdaltons) are both sensitive to degradation at high trypsin levels. A 23 Kd polypeptide of the light-harvesting pigment-protein complex serving PS I [18, 22] is altered in molecular weight by low trypsin levels. Almost all of the low molecular weight polypeptides (8−10 Kdalton size range) of PS I are degraded at high trypsin concentration; these polypeptides were previously identified as being required for electron transport steps on the reducing side of PS I [23].
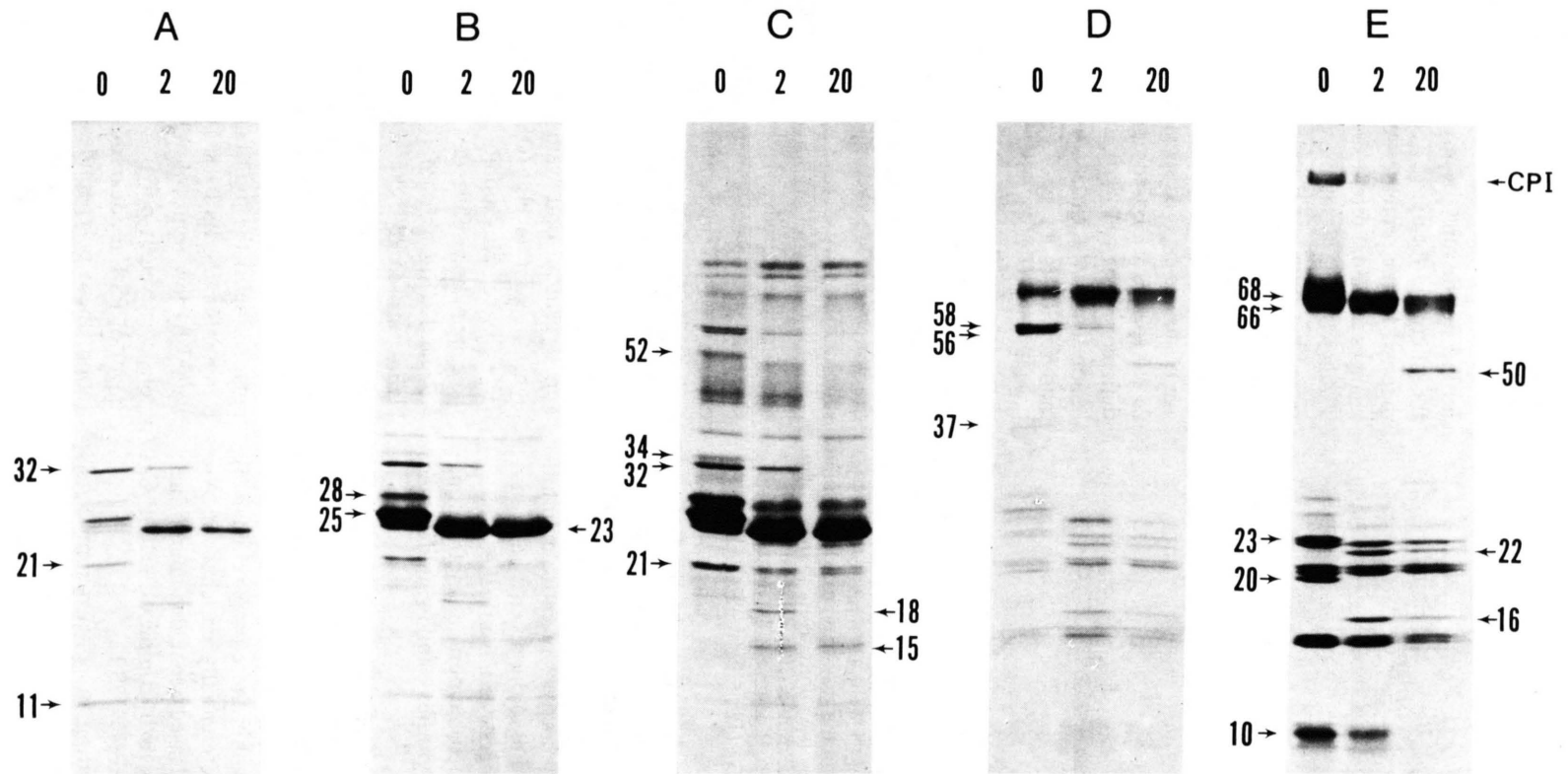
Fig. 9. Stained (Coomassie-blue) polyacrylamide gel of pea chloroplast thylakoids which were untreated (0), treated with 2 µg/ml trypsin (2), or 20 µg/ml trypsin (20) for 15 min. Fractions A through E are derived from detergent solubilized preparations which has been applied to sucrose density gradients (as described in legend of Fig. 8).

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The analysis of total thylakoid preparations after trypsin alteration of surface-exposed membrane proteins by SDS-PAGE has shown that at least 8 polypeptides are altered in molecular weight and are partially or totally removed the membranes even with "mild" (2 µg trypsin/ml) treatment conditions. In fractionated membranes, with higher resolution of separate polypeptide species of similar molecular weights, changes in 12 polypeptides were observed under the "mild" trypsin conditions. For consideration of PS II-directed herbicide receptors, however, the gradient system limits the candidates for trypsin-sensitivity to the polypeptides in Zone C of the gradient; *i. e.*, polypeptides of 52, 34, 32 and 20 Kdaltons.

Conclusions

Trypsin-treatment of chloroplast membranes affects atrazine affinity and the number of atrazine binding sites; similar effects on other herbicides are inferred from electron transport assays. Analysis of membrane proteins by SDS-PAGE revealed that at least four PS II-associated proteins are altered in a time period (15 min, 2 µg trypsin/ml) over which approximately 50% of the atrazine binding sites were lost. However, the components at 52 and 34 Kdaltons were already maximally altered (Fig. 9 C, lane 2). Since 50% of the sites are still present in this sample, it is improbably that these proteins contain the actual binding site, although their alteration may contribute to the change in herbicide affinity at another site within the PS II protein complex. The 32 Kdalton polypeptide of Fig. 9 C was partially decreased in staining intensity on the 2 µg trypsin/ml sample but was totally removed in the higher trypsin treatment (*i. e.*, when all binding sites were lost). This implicates the 32 Kdalton polypeptide as the atrazine receptor, in agreement with an earlier hypothesis of Croze *et al.* [7].

Our interpretations of trypsin effects, outlined above, are consistent with other recent studies in our laboratory. A photo-affinity triazine (azido-atrazine) has been found to bind covalently to the PS II-associated 34−32 Kd proteins [24]. Selective removal of the 32 Kd polypeptide from PS II particles results in loss of triazine sensitivity in PS II partial reactions [25]. In addition, chloroplasts from a maize mutant lacking the 32 Kd polypeptide [26] do not bind radioactive diuron (Leto and Arntzen, unpublished observations).

The present studies demonstrated that trypsin digestion of the 32 Kd polypeptide paralleled loss of herbicide binding sites and whole-chain electron transport activity. We suggest that this implicates the 32 Kd protein as being a functional component of the electron transport chain, perhaps serving as the apoprotein of B.

It is probably that the concept of an herbicide receptor region in the PS II complex will not be limited to understanding only one protein. Our observations on the differential affects of trypsin treatment on various herbicides (Fig. 6) are consistent with the idea that different "domains" within the PS II complex confer binding specificity to different chemical classes of PS II-directed herbicides [2, 27].

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