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Effect of trypsin on PS-II particles. Correlation between Hill-activity, Mn-abundance and peptide pattern

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The effect of trypsin treatment on Photosystem-II particles has been investigated by measurements of oxygen evolution, 2,6-dichlorophenolindophenol (DCIP)-reduction and Mn-abundance and by analyzing the peptide pattern. The following results were obtained. (1) Trypsin modifies both the acceptor and donor side of PS II, but striking differences are observed for the pH dependence: whereas the acceptor side is severely attacked between pH 5.5 and 9.0, the destruction of the donor side (oxygen-evolving capacity) by trypsin becomes significant only at pH values higher than 7.25. (2) The pH-dependence of the susceptibility of oxygen evolution to trypsin closely resembles that observed in inside-out thylakoids (Renger, G., Völker, M. and Weiss, W. (1984) Biochim. Biophys. Acta 766, 582–591). (3) The effect of trypsin on the functional integrity of water oxidation cannot be due to an attack on the surface exposed 16 kDa, 24 kDa and 33 kDa polypeptides, because they are digested rapidly even at pH 6.5, where the oxygen-evolving capacity remains almost unaffected. (4) Trypsination of PS-II particles as well as of the isolated 33 kDa protein leads to a 15 kDa fragment. In trypsinized PS-II particles this fragment remains membrane-bound. The amount of the 15 kDa fragment and Mn content are correlated with the oxygen-evolving capacity. These results indicate pH-dependent structural modifications at the donor side of System II which make target proteins accessible to trypsin. The 33 kDa protein is inferred to play a regulatory role in photosynthetic oxygen evolution and this function is realized by only a part of the protein, i.e., the 15 kDa fragment, that remains resistant to mild trypsination.

Introduction

Photosynthetic water cleavage in higher plants takes place in System II. This overall reaction can be described as plastoquinone reduction by electrons extracted from the ultimate donor, water, which becomes oxidized under proton release into molecular oxygen. The process of water oxidation is catalyzed by a special manganese-containing enzyme system (for a review, see Ref. 1). Whereas the overall reaction pattern is well-known as a four-step univalent redox reaction sequence, the mechanistic details are still far from understood. With regard to the role of polypeptides as apoenzymes or regulatory units, three proteins with molecular masses of approx. 16, 24 and 33 kDa have become a subject of extensive investigation in photosynthesis research, since the involvement of these proteins in water splitting was shown [2,3].

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Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethylbenzoquinone; DPC, 1,5-diphenylcarbazide; Mes, 4-morpholineethanesulfonic acid; PS, Photosystem; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

These polypeptides are located on the surface of the inner thylakoid membrane [4].

However, the exact function of these proteins is still a matter of debate [2,3,5–7]. One way to attack this problem is to analyze the effect of structurally selective and functionally specific proteolytic enzymes on the reaction pattern of photosynthetic water oxidation. As shown previously, trypsin is a useful tool in normal [8,9] as well as in inside-out thylakoids [10–12]. Trypsination of normal thylakoids specifically modifies the acceptor side of Photosystem II. It was found that trypsin digests a surface exposed protein acting as Q_A-Q_B apoprotein (because it is indispensable for electron transfer between the primary and secondary plastoquinone acceptors Q_A and Q_B). This protein also contains the binding site for DCMU-type herbicides and acts as barrier against exogeneous redox agents [8,9]. After mild trypsin treatment, the functional connection between Q_A and Q_B is interrupted: Q_A becomes directly accessible to exogeneous acceptors like $K_3Fe(CN)_6$ and the binding of DCMU-type herbicides is markedly decreased.

In contrast to that, trypsin treatment of thylakoids with inverted membrane polarity (inside-out vesicles) leads primarily to a destruction of the oxygen-evolving system [10,11] similar to the effect of Tris-washing, except for the dark-reduction kinetics of donor component D_1^{ox} [12]. In contrast to the proteolytic attack at the acceptor side in normal chloroplasts, the detrimental effect of trypsin on the water-oxidizing enzyme in inside-out vesicles reveals a strong pH dependence in the range of 6.5 to 7.4 [12]. This provides an invaluable discrimination from other types of trypsin-induced modifications.

Similar effects of trypsin on the water-oxidizing enzyme system are anticipated to arise in PS-II particles prepared by appropriate detergent treatment which permits retention of high oxygen-evolving capacity [13], because these membranes were inferred to contain a surface-exposed donor side of System II [14].

The present study was performed with special emphasis on a correlation between modifications of proteins involved in the water oxidizing system, manganese content and Hill-activity of PS-II particles.

Materials and Methods

PS-II particles with high oxygen-evolving capacity were prepared from market spinach as described in Ref. 13, with some modifications. Normal thylakoids, prepared by standard methods, were suspended (2 mg Chl/ml) in 400 mM sucrose/15 mM NaCl/5 mM $MgCl_2$/20 mM Mes-NaOH (pH 6.5) and incubated with Triton X-100 (50 mg/ml) on ice. This suspension was gently stirred and kept dark for 30 min. After a centrifugation step ($35\,000 \times g$; 20 min) the precipitate was resuspended in the above-mentioned buffer system without Triton X-100 and centrifuged at $5000 \times g$ for 10 min. Then the supernatant was spun down at $35\,000 \times g$ for 20 min, the pelleted PS II particles were suspended in the same buffer and stored in liquid nitrogen under adding of 30% ethylenglycol as cyroprotective agent. Inside-out vesicles from spinach were prepared as described in Ref. 15.

Before use, the preparation was thawed and washed twice with assay medium (300 mM sorbitol/10 mM NaCl/40 mM Mes-NaOH, pH 6.5) in order to remove nonspecifically released material. Bovine pancreas trypsin was purchased from Boehringer (Mannheim). The proteolytic treatment was performed for 15 min at room temperature at a chlorophyll concentration of 100 μg/ml. For the pH-dependent trypsin treatments the following buffers were used: Mes for the pH range 5.5–6.75, Hepes for pH 7.0 and Tricine for pH 7.25–9.0. The concentration of these buffers was 40 mM, and 10 mM NaCl and 300 mM sorbitol were added. In order to stop trypsin activity either trypsin inhibitor was used or samples were diluted with ice-cold buffer (300 mM sorbitol/10 mM NaCl/40 mM Mes-NaOH, pH 6.5), centrifuged ($35\,000 \times g$; 10 min) and subsequently washed twice with the same buffer. No difference in Hill-activity and Mn-abundance regardless of the way trypsin treatment was terminated could be detected. Due to the special treatment of samples digested for SDS-polyacrylamide gel electrophoresis (warming up to room temperature for 30 min after trypsination), one wash with a medium containing a high concentration of trypsin inhibitor (4-fold of trypsin) was applied prior to the twice washing in those experiments. After the last wash-

ing step, the samples were suspended in an appropriate buffer for further investigation. Samples for control experiments were treated in the same way without adding trypsin.

In order to remove the 16, 24 and 33 kDa proteins, either Tris-washing (0.8 M Tris-HCl, pH 8.35) or alkaline treatment (50 mM Tris-HCl/300 mM sorbitol/10 mM NaCl, pH 9.6) was performed. The particles were incubated for 30 min on ice, centrifuged, washed twice and suspended in assay medium. NaCl-washing for selective liberation of the 16 and 24 kDa proteins was done in a solution containing 1 M NaCl/40 mM Mes-NaOH/300 mM sorbitol (pH 6.5). After centrifugation the particles were washed and suspended in assay medium. The 33 kDa protein was isolated from PS-II particles. First the 16 and 24 kDa proteins were completely removed by two consecutive NaCl-washing steps as described above. Then the particles were suspended in assay medium containing 1 M $CaCl_2$, incubated for 50 min and centrifuged (35 000 × *g*, 20 min). In order to remove small fragments of PS-II particles, the resulting supernatant was again centrifuged (100 000 × *g*, 30 min) and subsequently desalted and concentrated with respect to the 33 kDa protein by two repetitive ultrafiltration steps (Amicon PM 10 membrane).

DCIP reduction was measured on highly diluted samples (5 μg chlorophyll/ml) with a Shimadzu UV-300 spectrophotometer at 600 nm in assay medium (pH 6.5) under addition of 35 μM DCIP. 18.0 $mM^{-1} \cdot cm^{-1}$ was used as extinction coefficient of the acceptor (pH 6.5).

When indicated in the figure legends, DPC (1 mM) and DCMU (10 μM) were present. Red saturating actinic light was used (Toshiba VR-65 filter). Control activities were: 200–250 and 250–350 μmol DCIP/mg Chl per h for the H_2O and DPC oxidation rates, respectively. Determination of oxygen evolution was performed with a Clark-type electrode at 25°C in assay medium (pH 6.5) with addition of either DMQ (1 mM) or $K_3Fe(CN)_6$ (2 mM)/$CaCl_2$ (10 mM). The chlorophyll concentration was 10 μg/ml. Manganese content was determined with a Shimadzu atomic absorption spectrophotometer (AA-640-13) equipped with a graphite furnace atomizer (GFA-3). 20 μl samples were dried at 150°C (50 s), gradually ashed between 150 and 700°C (75 s) and atomized at 2400°C (4 s).

SDS polyacrylamide gel electrophoresis was carried out in a slab gel apparatus using the buffer system of Chua [16] containing 5 M urea. Samples were dissolved in 2% SDS/10% mercaptoethanol/20% (w/v) sucrose/1 mM EDTA/10 mM Tris-HCl (pH 6.8), and analyzed using a slab gel containing 6% (stacking gel) and 16% (resolving gel) acrylamide. Gels were stained by Coomassie brilliant blue R-250 and analyzed with a Shimadzu CS-900 dualwavelength chromatoscanner.

Results

As PS-II particles with high oxygen-evolving capacity contain fragments of the partition membrane that do not form closed vesicles, trypsin should affect the acceptor side as in normal chloroplasts and the donor side in a similar way as in inside-out vesicles. Therefore, in the first step of this study we tried to clarify whether the suspected trypsin effect can be separated by phenomena that are caused by modification of the acceptor side and those arising due to a tryptic attack on polypeptides that affect directly (as apoenzyme) or indirectly (as regulatory units) reactions at the donor side with either H_2O or DPC as electron donor. Fig. 1 shows the results. With increasing concentration of trypsin in the incubation medium the electron transfer from H_2O to DCIP in PS-II particles becomes gradually inhibited, whereas the photoreduction of DCIP by DPC remains almost constant. If the same experiment is performed in the presence of DCMU at concentrations that suppress both control activities ($H_2O \rightarrow$ DCIP, DPC → DCIP) down to about 5 and 15%, respectively, this inhibition can be overcome for the most part by trypsination. If the applied trypsin concentration is high enough, both reactions ($H_2O \rightarrow$ DCIP, DPC → DCIP) are almost independent of the presence of DCMU. The DCIP reduction rates obtained under these conditions are only limited by the activity of the donor side (this leads to the small reduction rates for the reaction $H_2O \rightarrow$ DCIP).

The results depicted in Fig. 1 clearly demonstrate that in PS-II particles trypsin attacks the donor and the acceptor side. Tryptic digestion of

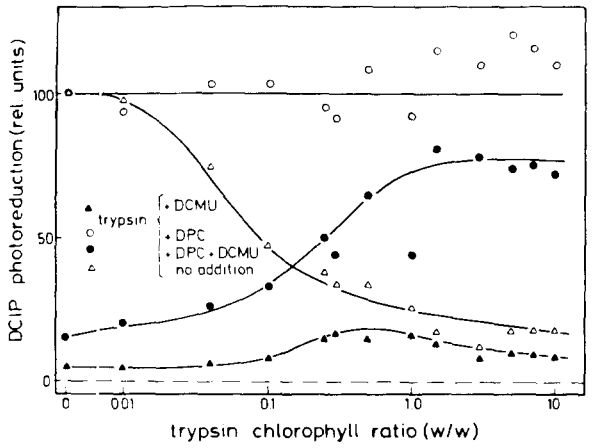

Fig. 1. The effect of trypsin on DCIP-photoreduction in PS-II particles. The proteolytic treatment was performed at pH 7.4. Other conditions as described in Materials and Methods.

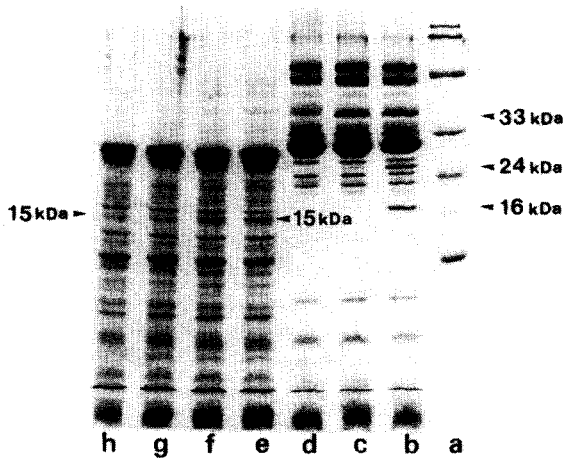

Fig. 2. SDS polyacrylamide gel electrophoresis electrophoretograms of trypsinized PS-II particles. (a) marker proteins; (b) control PS-II particles; (c) NaCl-washed; (d) Tris-washed; (e) trypsinized; (f) NaCl-washed and then trypsinized; (g) Tris-washed and then trypsinized; (h) trypsinized and then alkaline-treated. Trypsin digestion was done at 22°C for 15 min at pH 6.5 with trypsin/Chl (w/w) ratio of 0.5. A pair of arrows of 15 kDa fragment indicates its presence on (e) and (f), but its absence on (g) and (h).

the donor side leads to a decrease in Hill-activity, whereas at the acceptor side trypsin attacks the DCMU binding site without affecting the electron transfer to DCIP. The insensitivity of the DPC → DCIP electron transport to trypsin, which does not exist in normal chloroplasts [10,11], might reflect some modification of the acceptor side in PS-II particles, as outlined later in Discussion.

In order to characterize the trypsin-induced modifications at the protein level, SDS polyacrylamide gel electrophoresis was performed. Fig. 2 shows the results of this analysis. As can be seen in electrophoretograms (e) and (f), NaCl-washing prior to trypsin treatment did not induce any change in the polypeptide pattern of trypsinized PS-II particles. Since the NaCl-washed particles used in the present study contained no 16 and 24 kDa proteins (see electrophoretogram (c)), we may consider that both of these proteins were either liberated from the PS-II particles during the early stages of trypsin treatment or quickly digested into polypeptides of very low molecular weight which are beyond the detection range of the present polypeptide analysis. In this context it is noteworthy that the electron transport from H_2O to $K_3Fe(CN)_6$ shows a marked Ca^{2+}-effect (Table I). Since liberation of 24 kDa protein has been shown to enhance the Ca^{2+}-effect [21,22], the results of Table I favor the idea that neither the whole molecule of the 24 kDa protein nor its functional unit remained on the trypsinized PS-II particles.

Thus, all the multitude of polypeptide fragments appearing after trypsin treatment are considered to originate from protein components of the PS-II particles other than 16 and 24 kDa proteins.

Among these polypeptide fragments special attention should be paid to a 15 kDa polypeptide (arrow on electrophoretogram (e)), because this membrane bound unit can be removed by subsequent alkaline/Tris-washing (h), but not by NaCl-washing (data not shown), i.e., the membrane-binding properties of the 15 kDa fragment closely resembles those of the native 33 kDa protein. Accordingly, if the 15 kDa fragment were a digestion product of the surface exposed lysine-rich 33 kDa protein, it should not appear after trypsination of Tris- or alkaline-treated particles, since they are depleted of 16, 24 and 33 kDa proteins. This was found to be the case as shown by electrophoretogram (g) in Fig. 2 (see also Fig. 3d).

To make sure that the 15 kDa fragment is derived from the 33 kDa protein, purified 33 kDa protein isolated from PS-II particles was

TABLE I

Ca^{2+}-EFFECT ON OXYGEN EVOLUTION WITH FERRICYANIDE AS ACCEPTOR IN TRYPSINIZED PS-II PARTICLES

The proteolytic treatment was performed at pH 6.5. For details see text. No divalent cations were added. With DMQ as artificial electron acceptor no Ca^{2+}-effect in trypsin treated PS-II particles could be detected, because of the interrupted electron transfer to DMQ at the acceptor side of System II (vide infra).

PS-II particles	O_2-evolution (μmol O_2/mg Chl per h)		Mn abundance (atoms/400 Chl)
	$H_2O \rightarrow$ DMQ	$H_2O \rightarrow K_3Fe(CN)_6$	
Control particles	413	137	8.0
Trypsinized particles			
No addition	20	–	7.4
+ 10 mM $MgCl_2$	–	220	7.4
+ 10 mM $CaCl_2$	–	394	7.4
+ Alkaline-washing	–	12	0.3

trypsinized and peptide mapping was performed (Fig. 3). The digested samples, subjected to SDS polyacrylamide gel electrophoresis, again showed the presence of a 15 kDa fragment as clearly seen on electrophoretograms (e)–(i). From these results digestion of the 33 kDa protein by trypsin is confirmed to give rise to formation of a 15 kDa fragment which still remains associated with the membrane during trypsin treatment of PS-II particles because the essential binding determinants of the native 33 kDa protein are preserved in the fragment. In this context our observation is of note that the purified 33 kDa protein did not rebind at all to trypsinized PS-II particles, but partially (less than 10%) rebound when trypsinized particles were washed with 1 M $CaCl_2$ to remove the 15 kDa fragment.

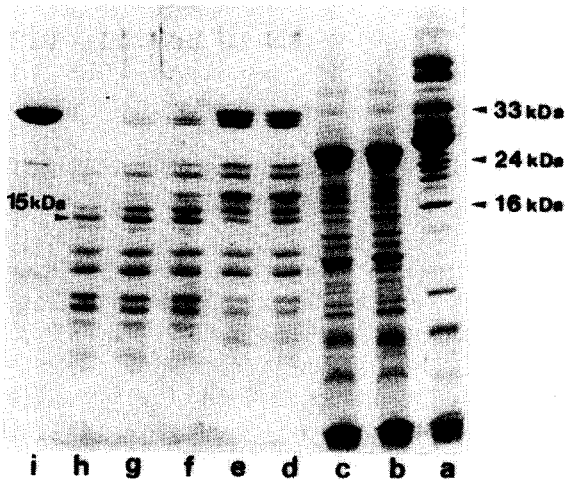

Fig. 3. Polypeptide mapping of the tryptic digestion products derived from purified 33 kDa protein. (a) control PS-II particles; (b) trypsinized PS-II particles; (c) trypsinized and then alkaline-washed PS-II particles; (d–h) trypsinized 33 kDa protein at increasing trypsin/33 kDa protein ratio from (d) to (h); (i) purified 33 kDa protein. Trypsin digestion was done at 22°C for 10 min at pH 6.5 with approx. trypsin/33 kDa protein ratio (w/w) of 0.002, 0.004, 0.006, 0.008 and 0.012 for (d)–(h), respectively. (c) is a repeated experiment of Fig. 2h.

Based on different lines of evidence, the 33 kDa protein was inferred to be an essential part of the water oxidizing enzyme system [2,3,6]. Therefore, a question arises to what extent the membrane-bound 15 kDa fragment remains able to satisfy the functional role of the native 33 kDa protein. Comparative measurements depicted in Fig. 4A show that tryptic digestion leads to a degradation of the intact 33 kDa protein at a rate much faster than the decrease in Hill-activity. (As the experiments were performed at constant incubation time with trypsin, the relative extent described in Fig. 4A reflects indirectly the degradation rate). Hence, the 33 kDa protein itself is not indispensable for sustaining the Hill-activity. It seems reasonable to assume that the 15 kDa fragment does not only contain the native binding site of the 33 kDa protein but also the active center (or essential structural elements) of this polypeptide and is therefore still able to perform the function of the intact 33 kDa protein (vide infra).

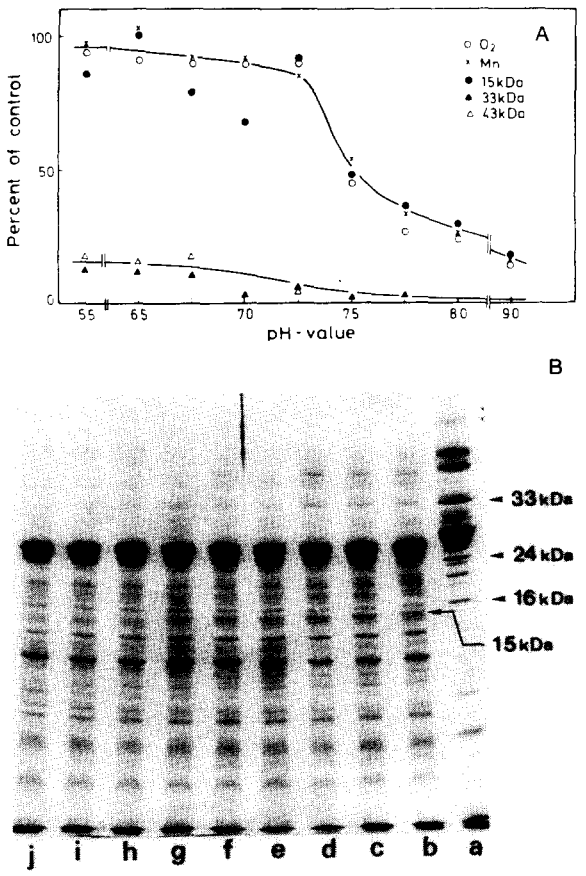

Fig. 4. (A) The effect of trypsin treatment on oxygen evolution, Mn abundance and protein content as function of pH in PS-II particles. The applied trypsin chlorophyll ratio was 0.5. Oxygen evolution was measured with $K_3Fe(CN)_6$ as acceptor in trypsinized particles and DMQ in control experiments (assay medium pH = 6.5). 100% control values were: 388 μmol O_2/mg Chl per h and 8.7 atoms Mn/400 Chl (control samples pH 6.5). The amount of proteins is given with respect to control samples pH 6.5, except the 15 kDa fragment, which is normalized on the amount appeared after trypsination at pH = 6.5. Other conditions as described in Materials and Methods. (B) SDS polyacrylamide gel electrophoresis electrophoretograms of PS-II particles after tryptic digestion at different pH values. (a) control PS-II particles; (b) pH 5.5; (c) pH 6.5; (d) pH 6.75; (e) pH 7.0; (f) pH 7.25; (g) pH 7.5; (h) pH 7.75; (i) pH 8.0; (j) pH 9.0. Densitometric determinations of 43 and 33 kDa proteins and of 15 kDa fragment of (A) were done with this electrophoretogram.

The impairment of the oxygen-evolving capacity by different treatments (Tris-washing, NH_2OH-treatment, etc.) often involves displacement and release of the catalytic manganese centers from their native environment (for review, see, e.g., Ref. 17). Therefore, it appears worthwhile to analyze the effect of trypsin and its pH dependence on the manganese content. The results depicted in Fig. 4A show that the trypsin-induced degradation of the oxygen-evolving capacity of PS II particles is accompanied by manganese loss. Both processes reveal a similar pH dependence, and the degree of inactivation of $H_2O \rightarrow K_3Fe(CN)_6$ electron transport closely corresponds to the relative amount of liberated manganese.

In order to exclude a pH-induced destruction of the oxygen-evolving system that is not related to trypsination, control experiments on non-trypsinised PS-II particles were performed. In these experiments PS II particles were treated (incubation, washing steps) under the same conditions, but without addition of trypsin. With DMQ as acceptor oxygen evolution was found to be constant between pH 5.5 and 8.0 and then steeply declines so that at pH 9.0 the control rate was only about 10% of that obtained at pH 6.5. Oxygen evolution with ferricyanide as electron acceptor revealed 40–50% of the activity obtained with DMQ at pH 6.5, regardless of the pH value (pH 5.5–9.0), therefore the oxygen-evolving activity mediated by DMQ at pH = 6.5 was used as 100% control value. The Mn content in control PS-II particles was pH-independent between pH 5.5 and 8.0.

The polypeptide maps of the PS-II particles trypsinized at different pH values are shown in Fig. 4B. The 15 kDa fragment could be clearly seen after trypsin treatment at pH ranges below 7.25, while it became obscure at pH 7.5 and almost disappeared at pH-values above 7.75. This pH-dependence also coincides well with those of manganese loss and inactivation of oxygen evolution. These results strongly support the idea that the pH-dependence arises from a modification of the donor side.

To confirm this conclusion, analogous experiments were performed with normal chloroplasts and inside-out vesicles, where trypsin attacks only the acceptor or the donor side, respectively. The data in Fig. 5 show that in normal chloroplasts trypsination does hardly affect the Mn content even at rather high trypsin concentration (similar data have been reported previously, Ref. 18),

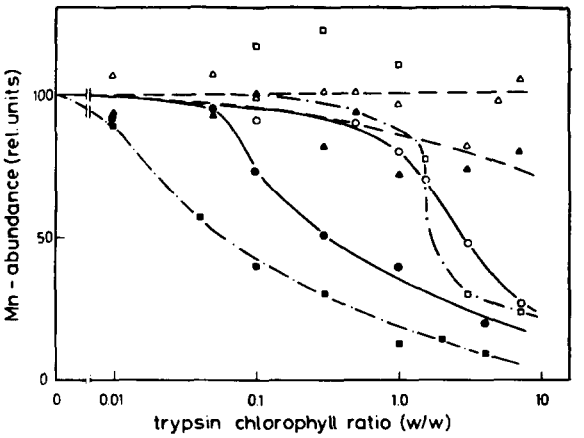

Fig. 5. Relative Mn abundance as function of the applied trypsin concentration in PS-II particles (□, ■), inside-out thylakoids (○, ●) and normal chloroplasts (△, ▲). Trypsin treatment was performed at pH 6.5 (open symbols) and pH = 7.4 (closed symbols).

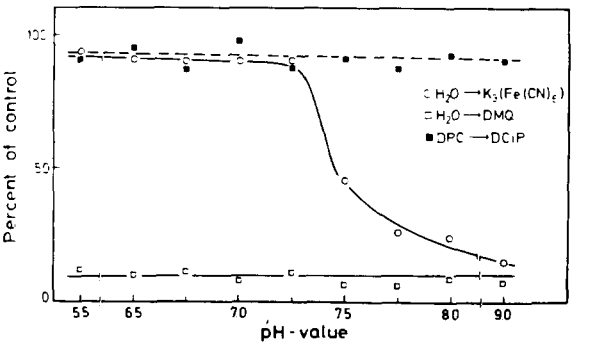

Fig. 6. The effect of trypsin on oxygen evolution and DPC → DCIP electron transport as function of pH in PS-II particles. Other conditions as in Fig. 4A. Results described in Figs. 4A, 4B and 6 were obtained in the same experiment. For further details see text.

whereas in inside-out vesicles a pH dependent manganese release is observed which resembles that of PS II particles. These findings prove that the mangenese release after trypsination is exclusively due to a modification of polypeptides that are exposed to the lumenal side of the thylakoid membrane and are functionally related to the function of the donor side.

As the relative oxygen-evolution rate in trypsinized particles correlates with the manganese content (Fig. 4A) as well as with the amount of the 15 kDa fragment, it appears reasonable to conclude that this part of the 33 kD aprotein is really able to satisfy the essential functional and structural requirements of the native polypeptide for sustaining water oxidation.

The above-mentioned considerations show that for the analysis of tryptic attack on the functional pattern of PS II particles, effects due to the acceptor and donor side have to be separated. As known from studies on inside-out vesicles, tryptic digestion of the donor side strongly depends on the pH value [11,12]. In contrast to that, the pH dependence of the trypsination pattern is rather small in normal thylakoids where primarily the acceptor side becomes modified [19]. Therefore, the different pH dependences of the susceptibility to trypsin offer a possibility for distinguishing the effects caused by the proteolytic attack of PS II particles at the donor and acceptor sides, respectively. So far, effects on the donor side were thoroughly described. In order to analyze the effect on the acceptor side, PS-II particles were trypsinized at various pH values (but constant trypsin concentration) and the oxygen-evolving activity was measured with different artificial electron acceptors.

In trypsin-treated normal thylakoids ferricyanide is able to accept electrons directly from the native primary quinone acceptor, Q_A, whereas the electron transfer to the endogeneous plastoquinone as well as to many artificial quinones is inhibited [8,19]. Fig. 6 reveals that a similar pattern arises after trypsination of PS-II particles. The Hill-activity with DMQ as acceptor is suppressed down to about 10% of the control value. This effect is almost pH independent, thus reflecting modifications at the acceptor side. In contrast to the foregoing, the oxygen-evolution rate of trypsinized PS-II particles with ferricyanide as exogeneous acceptor strictly depends on the pH. The results of Fig. 6 demonstrate clearly that at lower pH values the water-oxidizing enzyme system remains rather resistant to trypsin, so that in this pH range, trypsin effects on the acceptor side dominate the reaction pattern of PS-II particles. On the other hand, the requirement for higher pH values for successful tryptic modification of the donor side probably indicates essential pH-dependent structural rearrangements of target protein(s) at the donor side, making lysine and/or arginine residues accessible to trypsin (Figs. 4 and 6).

Discussion

The present study analyzing the trypsination pattern of PS II particles shows that modifying effects on the donor and acceptor side can be clearly distinguished by the pronounced pH dependence of the susceptibility of donor side polypeptides towards a tryptic attack.

With regard to the acceptor side it was shown that after trypsination the electron transport is strongly dependent on the nature of the exogeneous electron acceptors. Of special interest is the observation that synthetic quinones become rather poor electron acceptors after trypsination of PS II particles, whereas the efficiency of $K_3Fe(CN)_6$ remains unaffected. These effects closely resembling those observed after trypsination of normal chloroplasts [8,19] support the idea that a destruction of the structural integrity of the native binding site for plastoquinone in the Q_A-Q_B-apoprotein does not only prevent the intrinsic electron transport from Q_A to Q_B, but also eliminates the capability of synthetic quinones to act as electron acceptors [8,19]. An as yet not completely understandable difference to normal chloroplasts is the high resistance of DPC → DCIP electron transport in PS-II particles to trypsination (Figs. 1 and 6). This effect might be due to a slightly different trypsination pattern of the Q_A-Q_B-apoprotein in PS II particles that still permits full activity of DCIP reduction with DPC as donor, but already prevents synthetic quinones and endogeneous plastoquinone from reacting with Q_A^-, whereas, in normal chloroplasts, trypsination also impairs the electron transport to DCIP. Further experiments are required to clarify the details of this effect.

The main topic of this study, however, was the analysis of tryptic attack on polypeptides related to the function of the PS II donor side, especially of the water-oxidizing enzyme system. Various interesting findings were made.

(a) The recently in inside-out vesicles discovered strong pH dependence of a detrimental tryptic attack on the oxygen-evolving capacity [12] arises also in PS II particles and, therefore, appears to reflect structural changes at polypeptides that are closely related to the water-oxidizing enzyme system. These structural changes in the range of pH 6.5–7.8 do not affect the function of the water-oxidizing enzyme system but transfer it into a structural array which is highly susceptible to detrimental tryptic attack.

(b) The three surface-exposed proteins with molecular weights of 16000, 24000 and 33000 are removed or digested by trypsin at pH 6.5 and at 7.4, but oxygen evolution at pH 6.5 remains intact to a rather large extent. This shows that the structural integrity of neither of these proteins is indispensible for a functionally intact water-oxidizing enzyme system. At first glance this conclusion seems to be at variance with previous findings showing that NaCl-induced release of 16 and 24 kDa proteins causes a drastic reduction of the oxygen-evolving capacity [15]. When, however, we take into account that Ca^{2+} can be substituted for 24 kDa protein [21,22] and that our assay medium contained 10 mM $CaCl_2$ (see Materials and Methods), the resistance of oxygen evolution against removal of 24 (and 16) kDa protein(s) may be interpreted as partly due to the Ca^{2+}-effect, which would have compensated the activity reduction caused by the loss of 24 kDa protein. This idea may be supported by the result in Table I, where a marked Ca^{2+}-effect after trypsin treatment has been observed. Thus, it seems unlikely that a small part of the 24 kDa polypeptide, which survives the trypsination at pH 6.5 and escapes for its smallness our polypeptide analysis, is sufficient to sustain functional intactness of the water-oxidizing enzyme system. Obviously, the impairment of oxygen evolution (see Fig. 4) is mostly due to modification of the 33 kDa protein and manganese content, but is less or almost not due to modification of 24 and 16 kDa proteins.

(c) Trypsin cleavage of the lysine-rich surface exposed 33 kDa protein leads to a 15 kDa fragment that remains membrane-bound. This fragment is sufficient to sustain the structural requirements of the native environment of the catalytic manganese sites for manganese binding and functional activity. The 15 kDa fragment, however, cannot contain the catalytic site, because $CaCl_2$-induced removal of the native 33 kDa-polypeptide was already shown not to be coupled with manganese release [20]. Therefore, it appears reasonable to assume that the 15 kDa fragment plays the same regulatory role as the native 33 kDa unit.

It is very interesting, that the 15 kDa fragment

correlates in its pH-dependent susceptibility to tryptic attack with Mn binding and oxygen-evolving capacity. This effect could be explained in two different ways.

(i) The 33 kDa protein and its 15 kDa fragment act as 'shield' that prevents the catalytic Mn-binding environment from tryptic attack. In this case trypsin-induced distortion of the native catalytic site and concomitant Mn release would be limited by a pH-dependent degradation of the shielding protein. (ii) The 33 kDa protein and its 15 kDa fragment do not shield the Mn-binding site so that direct tryptic digestion of the native Mn-binding site and of the 15 kDa polypeptide are independent events with a similar pH dependence.

The latter explanation appears rather unlikely in the light of recent data of the $CaCl_2$ effect on manganese binding [20]. Therefore we suppose a shielding by the 33 kDa protein or its 15 kDa fragment of the catalytic Mn-containing site(s) in the water-oxidizing enzyme system. In this context it should be mentioned that an Mn release by tryptic digestion is not totally prevented at pH 6.5 but an approx. 30-fold higher trypsin concentration is required in order to obtain the same effect as at pH 7.4 (Fig. 5). This effect cannot be due to a pH-dependent increase of trypsin activity per se, because it amounts only a 50% stimulation in the above-mentioned pH range (see textbooks of biochemistry).

A very interesting phenomenon caused by trypsin treatment was the finding that the DPC → DCIP electron-transport activity was not affected after severe proteolytic treatment, where hardly any 43 and 47 kDa polypeptides could be detected by our SDS polyacrylamide gel electrophoresis technique. As the 47 kDa polypeptide is assumed to function as reaction-center-II apoprotein either smaller digestion fragments that are hardly stainable are still sufficient to support the reaction-centre-II photochemistry or the above-mentioned activity represents an artificial process. The latter idea seems to be less likely, because of the almost constant activity. Further experiments are required to clarify this very interesting point.

The data reported here and those of a recent study [12] indicate that PS-II particles and inside-out thylakoids reveal a similar behaviour towards trypsin treatment concerning the pH-dependent destruction of the water-oxidizing enzyme system.

The data in Ref. 12 can be explained more thoroughly in the light of the results presented in this study, if one accepts that the trypsination pattern of inside-out thylakoids is quite similar to that of PS-II oxygen-evolving membrane fragments. Tris washing or trypsination at pH 7.4 remove the 16, 24 and 33 kDa proteins (including the 15 kDa fragment) from the thylakoid membrane thus destroying the oxygen-evolving capacity and simultaneously modifying the electron-transport kinetics from D_1 to P-680$^+$. On the other hand trypsination of inside-out membranes or PS-II oxygen evolving fragments at pH 6.5 causes disappearance of the 16 and 24 kDa proteins together with the degradation of the 33 kDa protein down to its 15 kDa membrane bound fragment. This shows that the 15 kDa fragment plays a key role in sustaining the oxygen-evolving capacity as well as the mode of normal electron-transfer kinetics from D_1 to P-680$^+$. An additional effect of the 15 kDa fragment can be derived from the finding that trypsination of inside-out thylakoids deprived of their 16, 24 and 33 kDa (including its 15 kDa fragment) proteins (either by Tris-washing or by trypsin at pH 7.4) causes significant acceleration of D_1^{ox}-reduction by endogeneous component(s) [12]. Therefore an as yet unidentified protein, that effects the internal D_1^{ox}-reduction kinetics becomes susceptible to tryptic attack only after removal of the intact 33 kDa protein or of its 15 kDa fragment, i.e., this unit covers up the above-mentioned protein, which is digested by trypsin at either pH 6.5 or 7.4. The results presented here show that trypsin provides an invaluable tool to analyze the architecture of proteins that are related to the function of the water-oxidizing enzyme system.

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