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Localization in Photosystem II of the histidine residue putatively responsible for thermoluminescence A_1-band as probed by trypsin accessibility

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Effects of trypsin digestion on the capability of the thermoluminescence A_1-band that arises from charge recombination between Q_A^- and an oxidized histidine in PS II (Ono and Inoue (1991) FEBS Lett. 278, 183–186) were studied. The following results have been obtained. (i) Trypsin digestion markedly inhibited the capability of A_1-band only when applied at higher pH levels, showing a clear threshold pH at 7.25. (ii) This pH dependence agreed with that of the inhibition of O_2 evolution, but disagreed with the pH-independent modification of the acceptor side of PS II, the impairment of Q_A to Q_B electron transport. (iii) Trypsin digestion at pH 7.75 did not affect the capability of the EPR Signal II_f arising from oxidized secondary electron donor of PS II, even though it totally abolished the capabilities of both A_1-band and O_2 evolution. (iv) The susceptibility to trypsin of the A-band capability retained a pronounced pH dependence even after depletion of Mn, although the dependence curve was shifted to lower pH levels. (v) Trypsin digestion inhibited the capability of Mn^{2+}-photooxidation by Mn-depleted PS II, showing a pH dependence similar to those found for inhibitions of A_1-band capability and O_2 evolution. It was inferred that the histidine residue putatively responsible for the A_1-band capability is localized in the domain of a PS II protein(s) that provides ligands for the Mn-cluster, and this domain becomes exposed through pH-dependent structural rearrangement of the O_2-evolving enzyme to be attacked by trypsin.

Introduction

On the donor side of Photosystem (PS) II, a strong oxidizing equivalent produced by the primary photochemical reaction of P680 is transferred to Z (tyrosine 160 of D1 protein) and subsequently stabilized in the Mn-cluster consisting of four Mn atoms to oxidize water to molecular O_2 (for reviews see Refs. 1, 2). In addition to these components, D (tyrosine 161 of D2 protein), high-potential cytochrome *b*559 and chlorophyll are known to store oxidizing equivalents under some special conditions [3], although they do not participate in water cleavage, the main function of the donor side of PS II. Padhye et al. [4] have proposed that a histidine residue can operate between Z and the Mn-cluster as a redox active ligand of the Mn-cluster, and several lines of kinetic studies suggested that there will exist one more electron transport intermediate other than Z functioning between P680 and the Mn-cluster [5,6], although many ambiguities remain, due mainly to the difficulties in detecting a convincing signal from the candidate intermediate molecules.

Recently, new EPR and thermoluminescence signals were detected in Ca^{2+}-depleted PS II, and are proposed to be attributable to a new electron transport intermediate functioning on the donor side of PS II [7–9]. Based on its optical absorption spectrum and EPR spectrum, which assumes a magnetic coupling with Mn, the chemical entity of this new intermediate has been hypothesized to be a redox active amino acid residue (probably histidine) located in the close vicinity of the Mn-cluster [7]. A quite similar EPR signal was found in F^--substituted or Cl^--depleted PS II [10], and it was inferred that this redox species can be stabilized as the oxidized form only when the functioning of the Mn-cluster is impaired. When the Mn-cluster is depleted, both of these EPR and thermoluminescence signals are lost, but another thermoluminescence signal becomes detectable in place of these [11–13]. This thermoluminescence component (denoted as the A_1-

Abbreviations: D, auxiliary electron donor of Photosystem II; Z, secondary electron donor of PS II; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DPC, diphenylcarbazide; Q_A, primary quinone acceptor of Photosystem II; Q_B, secondary quinone acceptor of Photosystem II.

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band) is efficiently produced by illumination of Mn-depleted PS II at −20°C, and is suppressed by a very low concentration of exogenous Mn^{2+} [12]. The capability of A_1-band formation was reversibly inhibited by treatment with diethylpyrocarbonate that modifies histidine residue with high specificity [12]. These results are interpreted to mean that a histidine residue located in the vicinity of Mn-binding site is substantially photooxidized in Mn-depleted PS II. We also found that the putative histidine plays a role in photoligation of Mn^{2+} to reconstituted active Mn-cluster by functioning as a redox mediator between Z and exogenous Mn^{2+} [13].

In this communication, we report the effects of enzymatic digestion of PS II proteins by trypsin on various PS II activities including the A_1-band capability. It is known that in PS II membranes, the susceptibility of the O_2-evolving enzyme to proteolytic attack by trypsin shows a specific pH dependence: O_2 evolution is impaired only when digested above pH 7.5, while Q_A to Q_B electron transfer is equally impaired between pH 6.0 and 8.0 [14,15]. Based on these observations, it has been proposed that trypsin is accessible to the O_2-evolving enzyme only above pH 7.5, where the proteins on the donor side of PS II undergo structural rearrangement, probably due to deprotonation of basic amino acid residues [14,15]. It has been also reported that trypsinization degrades one of the two high-affinity Mn-binding sites that are sensitive to carboxyl amino-acid modifiers [16]. This information led us to apply trypsinization as a structural and functional probe selective for the protein domain near the O_2-evolving enzyme. It was found that A_1-band capability was lost by trypsinization in a pH-dependent manner identical to the loss of O_2 evolution and Mn^{2+}-photooxidation, while the EPR Signal II_f capability was not affected at any tested pH. Based on these data, we propose that both the putative histidine responsible for thermoluminescence A_1-band and the amino-acid residues ligating the Mn-cluster are located within the same domain of PS II proteins.

Materials and Methods

Triton X-100 solubilized BBY-type PS II membranes were prepared as described in Ref. 15, and stored in liquid N_2. Before use, the membranes were thawed and suspended in an assay buffer (0.4 M sucrose/40 mM Mes-NaOH/20 mM NaCl(pH 6.5)) after one wash with the same buffer. The PS II membranes were treated with 50 μg/ml TPCK-trypsin (Cooper Biochemical) in the dark for 20 min at 22°C at a chlorophyll concentration of 500 μg/ml. For pH-dependent trypsinization the following buffers were used: Mes-NaOH for pH 6.0–7.0, Hepes-NaOH for pH 7.25–7.5 and Tricine-NaOH for pH 7.75–8.5, all at the same concentration of 40 mM, including 400 mM sucrose and 20 mM NaCl. Trypsin digestion was stopped by dilution with a large volume of ice-cold assay buffer supplemented with soybean trypsin inhibitor (Type I-S, Sigma, 50 μg/ml). After two washes, the sample was resuspended in the assay buffer containing trypsin inhibitor (5 μg/ml) at a chlorophyll concentration of 2.5–4.5 mg/ml.

Removal of Mn was done by either NH_2OH treatment (3 mM NH_2OH/400 mM sucrose/40 mM Mes-NaOH/20 mM NaCl (pH 6.5)) or Tris treatment (0.8 M Tris-HCl (pH 8.7)) at 0°C for 30 min at a chlorophyll concentration of 250 μg Chl/ml. When indicated, PS II membranes were washed with 2 M NaCl before NH_2OH treatment for complete removal of 16 and 24 kDa peripheral proteins. The Mn-depleted membranes were centrifuged, washed once and suspended in the assay buffer.

DCIP-photoreduction was measured spectrophotometrically at 600 nm at room temperature in the assay buffer supplemented with 40 μM DCIP. When indicated, DPC (1 mM), DCMU (10 μM), $CaCl_2$ (20 mM) and $MnCl_2$ (70 or 10 μM) were added. O_2 evolution was measured with a Clark-type oxygen electrode at 25°C in the assay buffer supplemented with either dimethylbenzoquinone (2 mM) or ferricyanide (2 mM)/$CaCl_2$ (20 mM). DCMU (10 μM) was added when indicated. EPR Signals II_s and II_f were recorded at 20°C with a JEOL X-band EPR spectrometer model JES FE1XG as described earlier [13]. For thermoluminescence measurements, samples were excited by continuous light (> 500 nm) at −23°C or by a single flash light (white, 5 μs) at 5°C and frozen quickly in liquid N_2 and then the light emission during warming was recorded against sample temperature as described in Ref. 11.

Results

Fig. 1 shows the effects of trypsin digestion at various pH levels on two thermoluminescence components, the B-band and Q-band arising from charge recombination of $S_2Q_B^-$ and $S_2Q_A^-$ change pairs, respectively (for review see Ref. 17), and on the A_T-band that is proposed to arise from charge recombination between Q_A^- and oxidized histidine in Mn-depleted PS II [12]. PS II membranes were treated with trypsin and then thermoluminescence glow curves were recorded before and after depletion of Mn by NH_2OH treatment. Untreated Mn-retaining PS II membranes showed the B-band ($S_2Q_B^-$) at around 35°C after a single flash excitation. When digested with trypsin at pH 7.0, the peak temperature of the B-band was downshifted to around 18°C that coincided with the peak temperature of the Q-band ($S_2Q_A^-$), indicative of interruption by trypsinization of the electron transport from Q_A to Q_B. Note that the shoulder at around 0°C is an artifact due

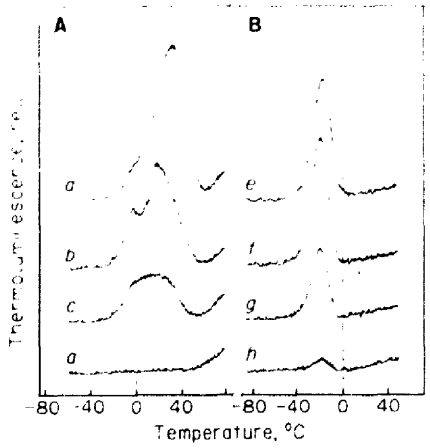

Fig. 1. Effects of trypsin digestion on thermoluminescence from Mn-retaining PS II and Mn-depleted PS II. O_2-evolving PS II membranes were trypsinized at the designated pH levels, and illuminated with a single flash at −4°C (panel A). For depletion of Mn, the trypsinized membranes were further treated with NH_2OH, and then illuminated with continuous light for 20 sec at −23°C (panel B). Trypsin digestion was done at 22°C for 20 min at three different pH levels at a trypsin/chlorophyll ratio of 0.1 (w/w). Non-trypsinized PS II membranes (a, e); PS II membranes trypsinized at pH 7.0 (b, f); pH 7.5 (c, g); pH 8.0 (d, h).

to the change in heating rate caused by melting of ice. After trypsinization at pH 7.5, the intensity of Q-band was largely suppressed, and after trypsinization at pH 8.0 the band was practically lost (panel A). Panel B shows the effect of trypsin digestion on the A_T-band. The trypsinized membranes and untreated control membranes were further treated with NH_2OH for complete depletion of Mn, and then excited by continuous light at −23°C for 20 s. Non-trypsinized but Mn-depleted membranes showed a strong A_T-band at around −20°C arising from charge recombination between Q_A^- and the putative oxidized histidine [12], but neither the B-band nor the Q-band was observed due to the absence of Mn. After trypsinization at pH 7.0 the A_T-band was normally induced, but after trypsinization at pH 7.5 its intensity was partially suppressed, and the band was almost lost after trypsinization at pH 8.0. Notably, the suppression of the A_T-band appears to be of all-or-none type: neither the shape nor the peak temperature of the glow curve was affected at all by trypsin digestion, indicating that the functioning of either the positive or negative charge carrier for this thermoluminescence component is specifically damaged, with no modulation in their redox properties (oxidation potential etc.).

The effects of trypsin digestion on various PS II activities are shown in Table I. O_2 evolution with dimethylbenzoquinone is largely inhibited after trypsinization at both pH 7.0 and 8.0, while O_2 evolution with ferricyanide was largely [14] enhanced after trypsinization at pH 7.0 but markedly inhibited after trypsinization at pH 8.0. It is of note that 80% of O_2 evolution with ferricyanide after trypsinization at pH 7.0 were insensitive to DCMU. DCIP-photoreduction with water was not much affected by trypsinization at pH 7.0 but was largely inhibited by trypsinization at pH 8.0. On the other hand DCIP-photoreduction with DPC was well retained regardless of the pH during trypsin digestion. Note that DCIP-photoreduction with DPC was measured with NH_2OH-treated samples in complete absence of Mn in order to avoid the interference due to the presence of Mn-cluster or exogenous Mn^{2+}. DCIP-photoreduction supported by DPC became DCMU insensitive after trypsin digestion (data not shown, see Ref. 14). These results indicate that both trypsinizations at pH 7.0 and 8.0 similarly modify the DCMU-binding site and induce more effective electron transport to ferricyanide than to synthetic benzoquinone acceptors, but trypsinization at pH 8.0 additionally affects the O_2-evolving enzyme consistent with our previous conclusion [15]. Based on these data, the results in Fig. 1 are interpreted as indicating that the capability of A_T-band formation is insensitive to damage of the Q_B site, but is highly sensitive to damage of the O_2-evolving enzyme.

Fig. 2 shows the effects of trypsin digestion at various pH levels on PS II activities. O_2 evolution with ferricyanide (open circles) and DCIP-photoreduction with water (open triangles) were not affected by trypsinization below pH 7.0, but declined steeply after trypsinization between pH 7.25 and 7.75, and was almost completely abolished after trypsinization above pH 8.0. The loss of O_2 evolution by trypsinization at higher pH levels is not due to a modification of the acceptor side, since DPC to DCIP electron transport

TABLE I

Effects of trypsinization on various PS II activities

PS II reactions	Activity [a] (μelectron equiv./mg Chl per h)		
	Non-trypsinized	Trypsinized [b]	
		pH 7.0	pH 8.0
H_2O → dimethyl benzoquinone [c]	2900 (690) [e]		
H_2O → ferricyanide [c]	310	1940 (1480) [e]	70
H_2O → DCIP [d]	540	610	14
DPC → DCIP [d]	690	710	710
A_T-band	100 [f]	194 [f]	7 [f]

[a] Electron transfer activities were measured at pH 6.5 in the presence of 20 mM $CaCl_2$.

[b] Trypsinization was done at 22°C for 20 min at a trypsin/Chl ratio of 0.1 (w/w).

[c] Measured by O_2 evolution.

[d] Measured by DCIP-photoreduction.

[e] DCMU (10 μM)-insensitive activity was indicated in parentheses.

[f] Intensity of A_T-band relative to that in nontrypsinized PS II was indicated.

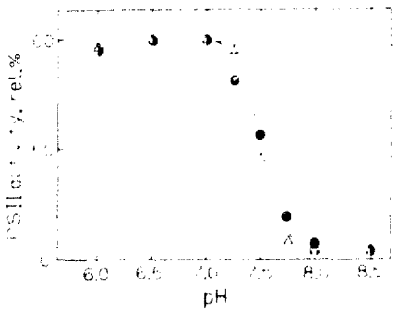

Fig. 2. pH-dependent inhibition by trypsinization of various PS II activities. O_2-evolving PS II membranes were trypsinized at various pH levels, and then subjected to assays of O_2-evolution with ferricyanide as electron acceptor (○) and DCIP-photoreduction with water as electron donor (△). The trypsinized membranes were further treated with NH_2OH to deplete Mn, and then subjected to assays of A_T-band capability (●) and DCIP-photoreduction with DPC as electron donor (▲). All assay buffers for electron transfer activities contained 20 mM $CaCl_2$. Conditions for trypsinization were the same as in Fig. 1.

(open squares) keeps a constant high level over all the pH range tested, while water to DCIP electron transport activity (open triangles) was inhibited specifically at higher pH levels. This clearly demonstrates the specific trypsinization of the proteins responsible for O_2 evolution at higher pH levels.

Fig. 2 also shows the effect of trypsinization at various pH levels on the capability of A_T-band. The A_T-band was constantly photoinduced after trypsinization below pH 7, but steeply inhibited after trypsinization between pH 7.25 and pH 7.75. The resulting pH-dependence curve was almost identical with those for inhibition of O_2 evolution. This suggests that the putative histidine, the positive charge carrier for A_T-band, is located in the O_2-evolving enzyme or implicated on a protein(s) adjoining the O_2-evolving enzyme, so that trypsinization of this protein(s) causes simultaneous loss of O_2 evolution and A_T-band capability.

If the above interpretation is correct, we may expect that destruction of the Mn-cluster will give rise to a conformational rearrangement of the target protein(s) and thereby alter the pH-dependent accessibility of trypsin. Fig. 3 shows the effect of Mn depletion on the inhibition of the A_T-band capability by trypsin digestion at various pH levels. PS II membranes were depleted of Mn with Tris or NaCl/NH_2OH treatment and then subjected to trypsin digestion. Note that Tris-treated membranes were devoid of all the three extrinsic proteins whereas NaCl/NH_2OH-treated membranes retained the 33 kDa extrinsic protein. Inhibition of the A_T-band capability by trypsin digestion revealed a pronounced pH dependency in Mn-depleted PS II membranes as well, but the dependence curve was slightly but significantly shifted to lower pH levels, showing a half-inactivation pH of about 7.5 for Mn-retaining PS II, and pH 7.2 for Mn-depleted PS II. This suggests that Mn depletion somehow facilitates the accessibility of trypsin to the protein(s) that bears the putative histidine, the positive charge carrier for the A_T-band. It is of note that the two pH-dependence curves obtained for Tris-treated and NaCl/NH_2OH-treated samples were identical to each other, regardless of the presence or absence of the 33 kDa protein. Probably, this protein is not a barrier for the attack by trypsin.

Fig. 4 shows the effect on EPR Signals II_s and II_f of trypsinization at pH 7.25 and 7.75. Trypsinized and non-trypsinized control Mn-retaining PS II membranes were further treated with NH_2OH for complete depletion of Mn, and EPR signals were measured in the presence of ferricyanide in order to keep Q_A oxidized by supporting multiple turnovers of the reaction center by accepting multiple electrons from Q_A^-. Note that the electron transfer between Q_A and Q_B is interrupted in trypsinized membranes (see Table I). In non-trypsinized membranes, illumination induced Signal II_f arising from Z^+, the oxidized form of the secondary electron donor of PS II, being superimposed on Signal II_s arising from D^+, the oxidized form of the auxiliary electron donor of PS II. Trypsinization at pH 7.25 did not affect Signals II_f and II_s, consistent with the finding that trypsinized membranes retained a high rate of O_2 evolution (see Fig. 2 and Table I). When trypsinized at pH 7.75, O_2 evolution and A_T-band capability were markedly suppressed (see Fig. 2), but more than 80% of Signals II_{s+f} could be detected, although the contribution from Signal II_s was much more decreased as compared with that in non-trypsinized membranes.

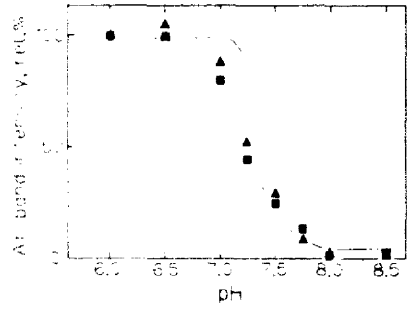

Fig. 3. Comparison of pH-dependent inhibition by trypsinization of A_T-band capability between Mn-retaining PS II and Mn-depleted PS II. For trypsinization of Mn-depleted PS II, O_2-evolving PS II membranes were first depleted of Mn by treatment with NH_2OH (▲) or with Tris (■), trypsinized at various pH levels, and then subjected to the assay of A_T-band capability. For trypsinization of Mn-retaining PS II, O_2-evolving PS II membranes were first trypsinized at various pHs, depleted of Mn by treatment with NH_2OH, and then assayed for A_T-band capability (○). The dependence curve with (○) is a reproduction of the corresponding curve in Fig. 2. The conditions for trypsinization and A_T-band induction were the same as in Fig. 1. A_T-band capability was expressed in percent relative to the intensity in non-trypsinized PS II.

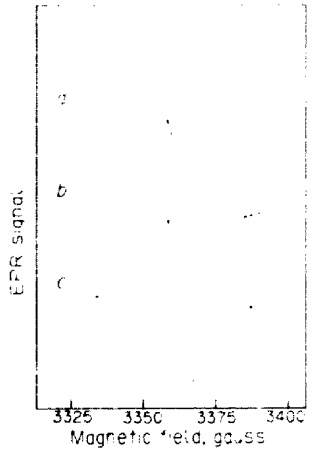

Fig. 4. Effects of trypsin digestion on EPR Signals II_s and II_f. Mn-retaining PS II membranes were trypsinized at 22°C for 20 min with a trypsin/chlorophyll ratio of 0.1 (w/w), treated with NH_2OH to deplete Mn, and then subjected to EPR measurement. Non-trypsinized control PS II membranes (a); PS II membranes trypsinized at pH 7.25 (b) and pH 7.75 (c). Solid curves indicate the light spectra recorded during illumination, and dashed curves indicate the dark spectra recorded in darkness following 1-min dark incubation after turning off the illumination. Chlorophyll concentration was 4.4 mg Chl/ml; Ferricyanide (2 mM) and $CaCl_2$ (20 mM) were supplemented in the assay buffer. Instrumental settings: microwave power, 1 mW; microwave frequency, 9.445 GHz; modulation frequency and amplitude, 100 KHz and 4 G, respectively.

These results indicate that Z is normally photooxidized even after trypsinization at pH 7.75. Völker et al. [18], however, reported that no Signal II could be photoinduced in PS II membranes trypsinized at pH 7.5. This discrepancy may have, arisen from the absence of an electron acceptor in their measurements: when only a single turnover is allowed, Z^+ decays rapidly through recombination with Q_A^-, since the Q_A to Q_B electron transfer is interrupted in trypsinized PS II. The low amplitude of Signal II_s in the membranes trypsinized at pH 7.75 is qualitatively consistent with the finding in Ref.18, and suggests that D^+ became more exposed to the ambient environment to be reduced more rapidly in the dark after illumination. Preservation of the capability of Z oxidation after trypsinization at pH 7.75 accounts well for the finding that DPC to DCIP electron transfer is not affected by trypsinization at any tested pH, as shown in Fig. 2: DPC will be efficiently oxidized by Z^+.

It has been reported that inactivation of O_2 evolution by trypsinization is accompanied by the release of Mn^{2+} from the O_2-evolving enzyme [15]. We attempted to estimate this destructive effect on the Mn-cluster by measuring the electron donation capability from exogenous Mn^{2+}. As shown in Fig. 5, the rate of Mn^{2+}-photooxidation was constant after trypsinization below pH 7.25, but was steeply decreased after trypsinization above pH 7.5 to reach a constant low level after trypsinization above pH 7.75. Note that the concentration of Mn^{2+} during the assay (75 μM) was controlled as low as the range of ligation affinity of Mn^{2+} during photoactivation [19,20]. The resulted pH dependence was quite similar to those for the capabilities of O_2 evolution and A_1-band formation with an identical half inhibition pH of 7.5. Assuming that the site of Mn^{2+} photooxidation is identical with the ligation site of the Mn-cluster, the results are interpreted to mean that trypsinization gives rise to damage of the Mn-ligation site, thereby leading to release of Mn and then inhibition of O_2 evolution. Notably, about 40% of the activity was retained after trypsinization at higher pH levels. Blubaugh and Cheniae [21] have reported that there are two types of Mn^{2+}-oxidation site on the donor side of PS II: for Mn ligation through photoactivation, the one with higher affinity is responsible. For Mn^{2+}-photooxidation, about 60% of the relative V_{max} value in the presence of 75 μM Mn^{2+} is due to the Mn^{2+} ions ligating at the high-affinity binding site, while the other 40% is ascribed to those at the low-affinity binding site. The trypsin-insensitive activity in our results may be attributable to the oxidation of Mn^{2+} binding to the low-affinity site.

There exists another very-high-affinity Mn^{2+}-binding site with a dissociation constant of the order of submicromolar [22–24], and binding of Mn^{2+} to this site has been proposed to competitively inhibit DPC-photooxidation [23]. As shown in Table II, the rate of DCIP-photoreduction with DPC by non-trypsinized, NH_2OH-treated PS II was inhibited by 40% by the addition of 10 μM $MnCl_2$, and similar extent of inhibition could be observed after trypsinization at pH 8.0 as well. This indicates that the site for very-high-affinity Mn-binding is insensitive to the trypsinization that destroys the Mn-cluster. Based on this observation, we hypothesize that the high-affinity Mn-binding site detected by DPC-DCIP competition assay may not be

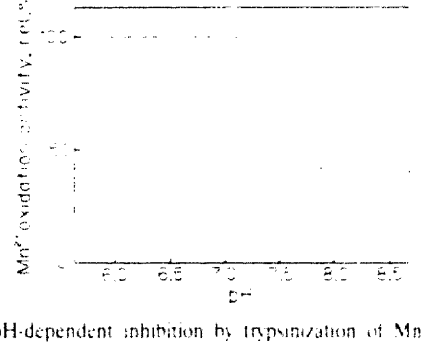

Fig. 5. pH-dependent inhibition by trypsinization of Mn^{2+}-photooxidation capability. Mn-retaining PS II membranes were trypsinized at various pH levels, then treated with NH_2OH to deplete Mn, and assayed for Mn^{2+}-photooxidation as measured by photoreduction of DCIP. Mn^{2+}-dependent activities were presented after subtraction of the basal activity (12 μequiv. mg Chl per h) observed in the absence of exogenous Mn^{2+}. The assay buffer contained 40 μM DCIP as electron acceptor and 75 μM $MnCl_2$ as electron donor.

TABLE II

Effect of trypsinization on Mn^{2+}-dependent suppression of DPC- to DCIP electron transport

Trypsinization was carried out at 22°C for 20 min at a trypsin/Chl ratio of 0.1 (w/w)

PS II membranes	DCIP-photoreduction (μmol DCIP/mg Chl per h) (relative)	
	no addition	+ 10 μM $MnCl_2$
Non-trypsinized	588 (100)	355 (60)
Trypsinized at pH 8.0	386 (100)	230 (60)

involved in ligation of the Mn-cluster. A relevant issue for this hypothesis is that DPC-photooxidation activity after trypsinization at pH 8.0 was inhibited by about 30% when assayed in the absence of Ca^{2+}, whereas it was not greatly inhibited in the presence of Ca^{2+} (see Table I and Fig. 2). The stimulation effect by Ca^{2+} of DPC-photooxidation might be related to the finding by Satoh et al. [25] that Ca^{2+} is functional as an indispensable cofactor for the electron transfer from Z to P680$^+$.

Discussion

The present study showed that the capabilities of thermoluminescence A_1-band and O_2 evolution were markedly inhibited by trypsin digestion with quite a similar pH dependency. The A_1-band capability and O_2 evolution were susceptible to trypsin digestion only above the threshold pH of 7.25, while the properties of PS II acceptor side were equally modulated between pH 6.0 and 8.5 (Figs. 1, 2). This indicates that, below this threshold pH, trypsin damages selectively the acceptor side of PS II leaving the O_2-evolving enzyme unaffected. When the acceptor side is damaged by trypsinization, the efficiency of electron acceptors for O_2 evolution changes dramatically: synthetic benzoquinones lose their high efficiency, while ferricyanide gains a very high efficiency. In contrast, the rate of DCIP-photoreduction remains unaffected (Fig. 2 and Table I). Based on the observation that binding of DCMU-type herbicides is severely affected [15,26], the main target of trypsinization below the threshold pH is believed to be the Q_B-site. Our observation in this study that the membranes digested at pH 7.0 exhibited thermoluminescence Q-band arising from $S_2Q_A^-$ charge recombination, indicating that Q_A, but not Q_B, is stably reduced (Fig. 1), is consistent with the previously proposed view [26,27] that mild trypsinization functionally disconnects Q_B from Q_A. Trypsinization at lower pH levels is also known to convert cytochrome *b*559 from high- to low-potential form [18]. Reasonably, however, this damage does not affect the capabilities of A_1-band and O_2 evolution (Fig. 1 and Table I), because the target components of the damage are not directly involved in either capability.

On elevating the pH during trypsin digestion, O_2 evolution becomes inhibited. This is interpreted that a pH-dependent structural rearrangement of PS II protein(s) leads the donor side of PS II to be accessible to trypsin [14,15]. After trypsinization above the threshold pH of 7.25, both O_2 evolution and A_1-band capability are concurrently inhibited (Fig. 2). The inhibition of O_2 evolution and A_T-band capability cannot be due to damage of redox functioning of Z, since the capability of Signal II_f arising from Z^+ is well retained after trypsinization at pH 7.75. This result is compatible with our previous proposal that the putative redox active histidine is functionally located between Z^+ and the Mn-cluster [13]. We may thus assume that trypsin digestion above the threshold pH specifically damages the structure and/or functioning of the O_2-evolving enzyme, the Mn-cluster among the various electron carriers on PS II donor side. Völker et al. [15] reported that release of Mn accompanies the trypsin-induced inhibition of O_2 evolution. Their finding appears to be compatible with our present results in that the binding site for exogenous Mn^{2+} that functions in photooxidation of Mn^{2+} in reconstitution of O_2 evolution was lost by trypsinization above pH 7.75 (Fig. 5). Based on these considerations, we speculate that trypsin at higher pH levels digests the proteinaceous domain responsible for Mn-ligation and this damage elicits inhibition of both the A_T-band capability and O_2 evolution.

We have to take into account, however, the possibility that the loss of A_T-band capability can also result from trypsin induced modification of Q_A properties that involves destabilization of Q_A^-: rapid dissipation of Q_A^- will decrease the A_T-band capability due to shortage in negative counterpart for charge recombination. However, this possibility is unlikely, based on the following results: (i) Q_A is stably reduced in right-side-out thylakoids after trypsinization at pH 7.5 (Ref. 28), suggesting that trypsinization at this pH specifically damages Q_B function, leaving Q_A function unaffected. (ii) DCIP photoreduction with DPC of NH_2OH-treated PS II retained high activity after trypsinization at every pH (Fig. 2). We may thus attribute the loss of A_T-band capability induced by trypsinization at higher pH levels to the damage on the PS II donor side. The idea that trypsinization at higher pH specifically affects the Mn-cluster can be supported indirectly by the results in Fig. 3 experiments that removal of Mn significantly altered the pH-dependence curve of trypsinization-induced inhibition of A_T-band capability. Probably, Mn removal gave rise to a subtle conformational change on the donor side of PS II, and thereby modulated the pH-dependent accessibility of trypsin to the Mn-cluster.

Based on these considerations, we presume that the putative histidine residue responsible for the A_1-band is located in, or in close vicinity of, the proteinaceous domain where the Mn-cluster resides, and is associated with the Mn-cluster in structural and functional terms. This presumption is compatible with our previous finding [13] that photodamage of the A_T-band capability in Mn-depleted PS II causes the loss of photoactivation capability to reconstitute the Mn-cluster through photooxidation of exogenous Mn^{2+} at its prospective ligation site. Renger et al. [14] have reported that trypsinization of Tris-treated inside-out thylakoids at pH 7.4 stimulates re-reduction of Z^+, and suggested that trypsinization induces a protein modification that causes rapid reduction of Z^+. If we assume that the putative histidine is functional as a redox intermediate between Z and Mn^{2+}, oxidizing equivalents will accumulate on the putative histidine in Tris-treated PS II, whereas they will accumulate on Z in Tris-/trypsin-treated PS II. Re-reduction of Z^+ will be more stimulated in the latter case, because of direct reduction of Z^+ by an ambient reductant due to the absence of the putative histidine.

We may reasonably assume that trypsin does not have access to proteins buried in lipid bilayer. In fact, trypsin digestion does not affect Z to Q_A electron transport which comprises only three redox components ligating to the amino-acid residues assumed in the membrane spanning segments of D1 and D2 proteins [29]. We may therefore assume that some arginine and/or lysine residues in the loops of D1 and/or D2 proteins protruding to the lumenal side of thylakoids are the target of trypsin attack that gives rise to the inhibition of A_T-band capability and thereby the loss of Mn-photoligation capability. According to the folding model of D1 and D2 proteins deduced from their DNA sequences [7,30], three trypsin sites can be pointed out on lumenal loops: one between helices I and II of both D1 and D2 proteins, one between helices III and IV of D2 protein, and one more on the C-terminal tail of both D1 and D2 proteins. Among these three sites, the structural disorder resulting from trypsinization will be most pronounced for the C-terminal tail of the two proteins, since maximally three polypeptide fragments are expected to be released by trypsinization, while no fragments come from the other lumenal loops. Notably, there exists a cluster of basic amino-acid residues including arginine and histidine in the C-terminal tail of D1 protein (e.g., R_{323} to H_{337}). At present we assume as a working hypothesis that protonation of this cluster will be responsible for the pH-dependent conformational rearrangement of the O_2-evolving enzyme and thereby to the increase in accessibility to trypsin. Recently, Nixon and Diner [31] have reported that site-directed mutation of Asp-342 and His-332 residues in the C-terminal tail of the D1 proteins abolishes the capability of O_2 evolution without impairing the electron transfer from Z to Q_B, and they have suggested that both Asp-342 and His-332 donate ligands to the Mn-cluster. Our working hypothesis appears compatible with their results.

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