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STUDIES ON THE NATURE OF THE WATER-OXIDIZING ENZYME

I. THE EFFECT OF TRYPSIN ON THE SYSTEM-II REACTION PATTERN IN INSIDE-OUT THYLAKOIDS

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The effect of mild trypsination on the system-II reaction pattern of inside-out thylakoids has been analyzed by measurements of oxygen yield, fluorescence induction and laser-pulse-induced absorption changes at 320 and 830 nm. The following was found. (1) The average oxygen yield per flash drastically declines after trypsination at pH 7.4, while at pH 6.5 only small effects are observed. (2) The area over the fluorescence induction curves becomes reduced by 30–40% after trypsination at either pH 6.5 or 7.4, but in the latter case the maximum level is attained only after addition of hydroxylamine as PS-II donor. On the other hand, the area over the induction curve in the presence of DCMU, which is 10–12 times smaller than without DCMU, remains unaffected by trypsin treatment. (3) The oscillation pattern of the oxygen yield induced by a flash train in dark-adapted inside-out thylakoids is not markedly affected by trypsin treatment, even at more than 80% inhibition of the oxygen-evolving capacity. (4) After trypsination of inside-out thylakoids, a large 10 μs decay arises in the relaxation kinetics of the 830 nm absorption changes, whereas the 320 nm absorption changes are dominated by a rather slow decay. (5) The half-life time of the microsecond kinetics at 830 nm elicited by trypsination of inside-out thylakoids reveals almost the same pH dependence as the corresponding relaxation kinetics in Tris-washed inside-out thylakoids. (6) The relaxation kinetics of the absorption changes at 320 and 830 nm in Tris-washed inside-out thylakoids become significantly modified after trypsin treatment. Based upon these findings, it is concluded that beyond the well-characterized polypeptides with 16, 23 and 33 kDa there exists a further protein that is exposed to the inner side of the thylakoid and that affects the electron transport in system II. The nature and the physiological role of this polypeptide still remain to be elucidated.

Introduction

The reaction sequence of photosynthetic water-cleavage taking place in system II can be summarized as plastoquinone reduction with water as electron-donor driven by electron ejection from the lowest excited singlet state of a special chlorophyll-*a* complex, referred to as P-680. The overall

Abbreviations: D_1, redox component connecting P-680 and the water-oxidizing enzyme system Y; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl-1,1-dimethylurea; Mes, 4-morpholineethanesulphonic acid; DPC, 1,5-diphenylcarbazide; PS II, Photosystem II; P-680, photoactive chlorophyll of Photosystem II; Q_A and Q_B, primary and secondary plastoquinone of system II, respectively, Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

process gives rise to oxygen evolution, plastoquinol formation and the generation of a transmembrane pH difference. Water photolysis is realized by a complex of redox enzymes that are anisotropically embedded into the thylakoid membrane (for recent review, see Refs. 1 and 2). The kinetic pattern of the reaction sequence is well resolved, less information is available on the nature of the functional redox groups and almost nothing is known about the structure and mechanism of the indispensable apoenzymes that regulate the kinetic and energetic properties of the different redox enzymes. Selective proteolysis appears to be a promising tool for unraveling the function of protein matrices acting as apoenzymes. In this way, modifications of the reaction pattern induced by mild trypsination of normal chloroplasts led to the conclusion that special protein(s) exposed to the outer surface of the thylakoid membrane are indispensable for the electron transfer between the primary and secondary plastoquinone, Q_A and Q_B, respectively, at the acceptor side of system II, and, simultaneously, act as barrier to the transport of H^+ and exogenous redox agents as well as containing binding sites for most PS II herbicides [3,4].

Most of the redox enzymes catalyzing water oxidation by P-680$^+$ are exposed to the inner surface of the thylakoids [5,6]. Accordingly, inside-out thylakoids with inverted membrane polarity are especially suited for analysis of the functional role of proteins that act directly as apoenzymes or indirectly as regulatory proteins for the functional groups required for water oxidation. It was found that three polypeptides of 16, 23 and 33 kDa are involved [5,7], and especially the 23 kDa one was shown to be required for the functional connection between the water-oxidizing enzyme system Y and P-680 via the donor component, D_1, [8] probably as a structurally essential factor rather than directly participating in electron transport. Because all of the three polypeptides contain a significant amount of lysine and some arginine [7,9], they should be susceptible to tryptic digestion. Indeed, the electron-transport activity with H_2O as donor degrades during mild trypsin treatment without affecting the donor function of DPC [10] and the functional connection of D_1 with P-680 in Tris-washed inside-out thylakoids [11].

Even more interesting, latest data showed that in inside-out thylakoids the effect of trypsin is quite different at pH 7.4 from that observed after trypsination at pH 6.5 [12]. Previous reports were lacking a systematic analysis of the PS-II reaction pattern. Therefore, the present study was performed in order to attempt to correlate the structural modifications caused by mild trypsination of inside-out thylakoids with their effects on the reaction pattern of system II.

Materials and Methods

Inside-out thylakoids were prepared by mechanical disintegration of class-II chloroplasts followed by aqueous two-phase fractionation as previously described [13]. Tris-washing of inside-out thylakoids was performed according to the Yamashita-Butler procedure [14]. Trypsination was analogous to that described in Ref. 14. (The trypsin/chlorophyll ratio is indicated in figure legends.) The standard reaction mixture contained: chloroplasts (5, 10 and 50 μM chlorophyll for measurements of fluorescence induction, absorption changes and average oxygen-yield measurements, respectively), 10 mM KCl, 2 mM $MgCl_2$, 50 μM phenyl-*p*-benzoquinone as electron acceptor and either 20 mM (Mes)-NaOH (pH = 6.5) or Tricine-NaOH (pH = 7.4).

Absorption changes at 320 nm of dark-adapted chloroplasts were measured by using a pulsed measuring light beam as described in Ref. 16. Measurements at 830 nm were performed analogous to those in Ref. 11.

The oxygen yield under repetitive flash excitation was measured with a Clark-type electrode as outlined in Ref. 17, the oscillation pattern in dark-adapted chloroplasts was detected with an unmodulated Joliot-type electrode [18] as described in detail in Ref. 19. Fluorescence induction curves were measured as in Ref. 12, using blue actinic light.

Results

Based on measurements of DCIP reduction under continuous saturating light, mild trypsination at pH 7.4 was inferred to attack selectively the oxygen-evolving capacity [10]. This effect could

either be due to retardation of an electron-transfer step at the donor side or due to a complete destruction of the water-oxidizing enzyme system Y or its disconnection from the reaction center. In order to eliminate possible kinetic effects and to measure directly the number of functionally intact systems II, the stationary oxygen yield was determined under repetitive flash excitation. The results obtained are depicted in Fig. 1. They reveal that trypsination at pH 7.4 causes a rapid decline in oxygen yield which directly reflects the number of functionally active systems II. The oxygen-evolving capacity exhibits a clear biphasic decline indicating that approx. 30% of the total systems are more resistant to trypsin. This phenomenon might reflect some heterogeneity of the donor side components to tryptic attack because it cannot be explained simply by contamination of right-side out particles which were found to be even more susceptible to proteolytic degradation at the acceptor side under these conditions (Ref. 10 and Völker, M. and Renger, G., unpublished data). The complete blockage of electron transport by DCMU further supports this idea. Preliminary data (Völker, M. and Renger, G., unpublished data) also reveal that the donor side of PS II in inside-out thylakoids becomes affected by a lysine-specific

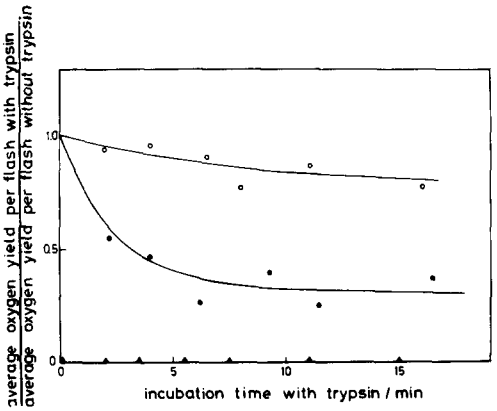

Fig. 1. Average oxygen yield per flash in inside-out thylakoids as a function of incubation time with trypsin related to the corresponding control value without trypsin. The incubation medium in the cuvette contained 15 μg trypsin/ml and 50 μg chlorophyll/ml (ratio, 0.3 : 1) Other conditions as in Materials and Methods. Time, t_d, between flashes, 500 ms; flash duration, approx. 15 μs. (○), pH 6.5; (●), pH 7.4, without DCMU; (▲), pH 7.4, with 3 μM DCMU.

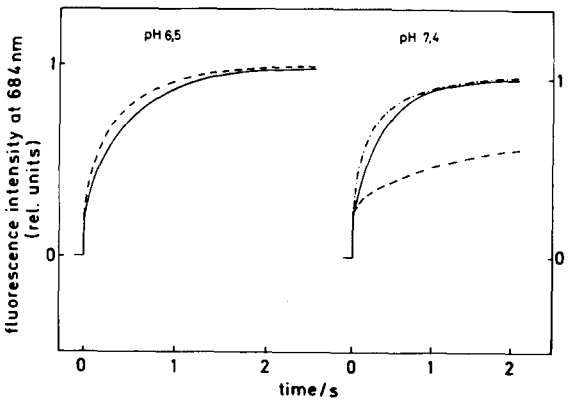

Fig. 2. Fluorescence induction in inside-out chloroplasts. Trypsinized inside-out thylakoids were treated for 15 min at a ratio of 0.3 : 1 (15 μg trypsin per ml/50 μg chlorophyll per ml) and diluted before the onset of the measurements to 5 μg chlorophyll/ml; (- - - - -), trypsin; (· – · – ·), trypsin + NH_2OH. Control samples (———) were treated in the same manner but without trypsin. Other details, see Materials and Methods.

proteinase, while an arginine specific protease appears to be completely inactive. A striking phenomenon, however, is the almost complete resistance if the mild trypsin treatment is performed at pH 6.5 under otherwise identical experimental conditions. This agrees with recent results observed for the DCIP reduction [12]. The effect cannot simply be due to the pH dependence of trypsin activity, because it is only twice as high at pH 7.4 compared to pH 6.5 (see textbooks on Biochemistry). This conclusion is also confirmed by the effect of trypsin on the acceptor side of PS II in normal chloroplasts [20]. Further experimental evidence for the strong pH dependence of trypsination in inside-out thylakoids to be restricted to the donor side of system II, is the effect on the fluorescence induction curves. The results in Fig. 2 show that trypsination at pH 6.5 reduces the area over the induction curve by approx. 30%, whereas the induction curve in the presence of DCMU remains unaffected. In control inside-out thylakoids, the ratio of the areas over the induction curves in the absence and presence of DCMU, respectively, is of the order of 10–12, indicating a slight reduction of the total pool size in inside-out thylakoids compared to normal class-II chloroplasts. Furthermore, the pool reoxidation in the dark was found to be slower than in normal chloroplasts (data not shown).

The possibility that contamination by right-side-out particles could be responsible for the observed effect on the fluorescence induction curve appears rather unlikely, because trypsination of normal chloroplasts gives rise to a drastic reduction of the fluorescence maximum level [21]. Therefore, as the maximum level remains unaffected, the results at pH 6.5 suggest that there exists a polypeptide exposed to the innner side that affects the overall pool size. This polypeptide has not yet been identified but one could speculate that the cytochrome b_6-f complex is affected, thereby reducing the electron acceptor capacity.

The reduction of the total pool size is also caused by trypsination at pH 7.4, but additionally the fluorescence does not attain its maximum level due to the destruction of the oxygen-evolving capacity under these circumstances. Addition of NH_2OH as PS-II donor restores the maximum level of the fluorescence induction curve.

The above-mentioned considerations led to the conclusion that the strong pH dependent decline in the oxygen-evolving capacity caused by mild trypsination of inside-out thylakoids (see Fig. 1), is probably due to conformational changes in proteins at the donor side that modify the exposure of lysine and/or arginine residues to tryptic attack. Therefore, questions arise about the nature of these proteinaceous components.

Three polypetides with 16, 23 and 33 kDa appear to be the most likely candidates, because: (a) they are claimed to be required for intact oxygen-evolving capacity [5–10, 22]; (b) they do contain lysine and arginine [7,9]; and (c) they are exposed to the thylakoid lumen side and therefore easily accessible to trypsin in inside-out thylakoids. The sharp difference in the trypsination pattern at pH 6.5 and 7.4, respectively, might suggest the involvement of a specific proteinaceous component. Among the three polypeptides the 23 kDa species appears to be interesting in this respect, because its isoelectric point was reported to be in the range of pH 6.5–7.3 [7,9,23]. Accordingly, a change in the net charge of the 23 kDa polypeptide could give rise to different susceptibilities to a tryptic attack at the donor side. In order to test this idea, the oscillation pattern of the oxygen yield induced by a flash train of dark-adapted inside-out thylakoids was measured, because these

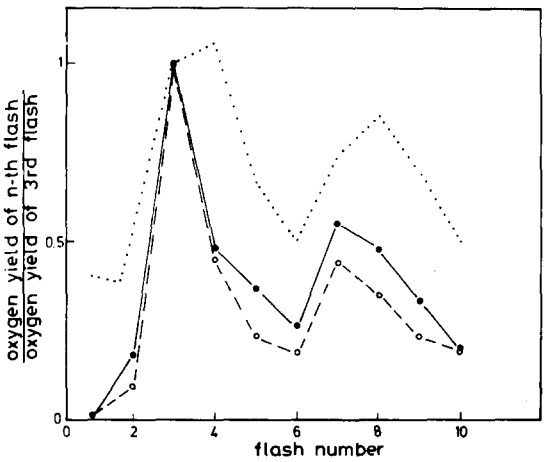

Fig. 3. Oxygen yield per flash in inside-out thylakoids normalized to the yield of the 3rd flash. Chloroplasts were treated at pH 7.4 with trypsin (●) for 10 min. at a 1 : 1 ratio (1 mg trypsin per ml/1 mg chlorophyll per ml) before starting the measurements. Other details see Materials and Methods. The data for salt-washed inside-out thylakoids ····· were taken from Ref. 24. ○, Control.

characteristics were recently shown to become markedly modified after selective removal of the 23 kDa polypeptide (together with the 16 kDa unit) by salt treatment. The data shown in Fig. 3 reveal that, in contrast to salt washing, the characteristic oscillation is not significantly altered after a severe trypsin treatment (1 mg trypsin per 1 mg chlorophyll at pH 7.4 for 10 min) that causes the blockage of more than 80% of all systems Y (deduced from the signal amplitude due to the 3rd flash of the train). Accordingly, a specific tryptic attack including only the 23 kDa polypeptide appears rather unlikely. The results reported so far rather favor the idea that trypsin does not only modify the 23 kDa and probably the 16 kDa polypeptide, but also reacts with the 33 kDa protein. This conclusion is in line with previous findings [25] indicating a complete digestion of this polypeptide at alkaline pH. Therefore, trypsinized inside-out thylakoids are expected to reveal similar properties as Tris-washed inside-out thylakoids, because Tris-washing was found to cause depletion of all three polypeptides in inside-out thylakoids [26] as well as in PS-II preparations [6,7].

In order to characterize the trypsin effect more thoroughly, absorption changes were measured at 320 and 830 nm, reflecting the turnover of the

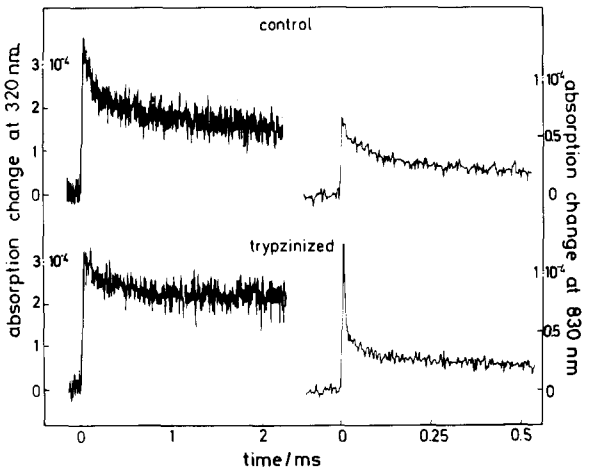

Fig. 4. Absorption changes at 320 and 830 nm as a function of time in inside-out thylakoids. Trypsination was performed for 20 min at pH 7.4 and at a 1 : 1 ratio (10 μg trypsin per ml/10 μg chlorophyll per ml). Chloroplasts were excited with a repetitive pulse from an Nd-YAG-laser (duration, 8 ns). Time, t_d, between the flashes, 1 s. Other conditions as described in Materials and Methods.

PS-II acceptor side as well as the water-oxidizing enzyme system Y and P-680, respectively. Absorption changes at 320 and 830 nm measured under repetitive laser pulse excitation in control and trypsinized inside-out thylakoids in the presence of phenyl-*p*-benzoquinone as exogeneous electron acceptor are depicted in Fig. 4. The relaxation kinetics of the 320 nm absorption change in control inside-out thylakoids can be described by a triphasic decay with half-life times of the order of 100 μs or less, of 1 ms and with slower kinetics. The 1 ms kinetics which amount to about 20% of the total initial amplitude is ascribed to the turnover of the water-oxidizing enzyme system Y reflecting the transition $D_1^{ox}S_3 \rightarrow (S_4) \rightarrow D_1S_0 + O_2$ [16]. The large extent of the slow kinetics probably reflects the reoxidation between the flashes of the semireduced secondary plastoquinone Q_B^- by the exogeneous acceptor. For the moment we cannot offer a simple interpretation for the $\leqslant 100$ μs kinetics (vide infra). After trypsination at pH 7.4 leading to severe destruction of the oxygen-evolving capacity, the total initial amplitudes become diminished by approx. 20% and the relaxation kinetics are characterized by a predominant (70–75%) slow decay and a minor 500 μs component. The slightly smaller initial amplitude could be explained by an elimination of the system Y turnover, similar to that induced by Tris washing. However, the relaxation kinetics of the 320 nm absorption changes in trypsinized normal inside-out thylakoids are quite different compared to those in Tris-washed normal chloroplasts [27] or Tris-washed inside-out thylakoids (see Fig. 6).

In control inside-out thylakoids the relaxation of the 830 nm absorption changes exhibits only a slow phase together with a comparatively small contribution of microsecond kinetics; whereas the predominant nanosecond kinetics [28] could not be resolved with our measuring device. After trypsin treatment, however, large decay kinetics with approx. 10 μs half-life time arise. These kinetics are indicative of the electron transfer from donor D_1 to $P-680^+$ in Tris-washed normal [27] or Tris-washed inside-out [11] thylakoids. Therefore, the 830 nm measurements of Fig. 4 suggest that trypsin might affect the P-680-D_1 electron transfer in a similar manner as other procedures which destroy the water-oxidizing enzyme system Y. In order to test this idea, the fast microsecond kinetics elicited by trypsination of inside-out thylakoids was measured as a function of pH because, regardless of the membrane polarity, these kinetics reveal a characteristic pH dependence in the 5–20 μs

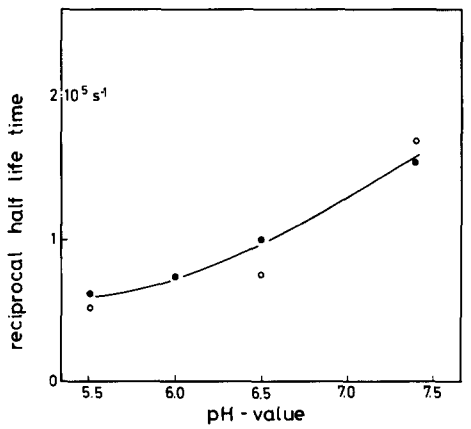

Fig. 5. Reciprocal half-life time of the transient 830 nm absorption changes in trypsinized inside-out thylakoids as a function of pH. Inside-out thylakoids were trypsinized (●) at pH 7.4 and at a ratio of 1 : 1 (10 μg trypsin per ml/10 μg chlorophyll per ml) for 10 min before changing the pH of the suspension to the indicated values. (○), Tris-washed inside-out thylakoids. Other experimental conditions as described in Fig. 4.

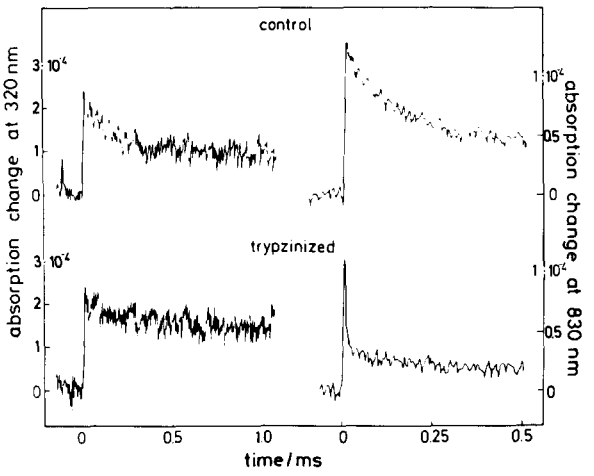

Fig. 6. Absorption changes at 320 and 830 nm as function of time in Tris-washed inside-out thylakoids. Repetition rate, 84 Hz; other experimental conditions as in Fig. 4.

range in Tris-washed thylakoids [11,29].

It was found that in accordance with the resistance of oxygen-evolving capacity (see Fig. 1) trypsination at pH 6.5 does not lead to the appearance of these kinetics even after prolonged treatment (data not shown). In order to measure the pH dependence of trypsin-induced decay kinetics, inside-out thylakoids were trypsinized at pH 7.4 and afterwards transferred to a buffer suspension of desired pH. The results depicted in Fig. 5 reveal that the pH dependence and the absolute half-life times of the 830 nm decay kinetics in trypsinized chloroplasts closely resemble those observed in Tris-washed normal [29] or inside-out thylakoids (Völker, M., diploma thesis, Berlin, 1982). Therefore, the kinetics very likely reflect the electron transfer from D_1 to P-680$^+$, i.e., trypsination of inside-out thylakoids at pH 7.4 affects this reaction in a similar way as Tris-washing and other comparable procedures. However, there does exist a striking difference to Tris-washed thylakoids. In trypsinized inside-out thylakoids, the 5–20 μs kinetics are observed under comparatively high-frequency rates of the laser pulses (10 Hz), whereas in Tris-washed thylakoids under these excitation conditions the back reaction between Q_A^- and P-680$^+$ characterized by 100–200 μs half-life time [11,27,30] dominates the 830 nm relaxation kinetics because the D_1 recovery is significantly slower. Therefore, the difference in kinetical behavior of Tris-washed and trypsinized inside-out thylakoids can be easily explained by the assumption that D_1 becomes much faster reduced in the latter thylakoids. This interpretation is in line with the rather slow recovery of the 320 nm absorption changes. Two alternative mechanisms could be responsible for the different reaction patterns: (a) trypsin treatment causes only a partial structural modification of polypeptides which is sufficient to destroy the capability for water oxidation, but simultaneously favors the fast recovery of D_1 by electron donation from the acceptor side, (b) there does exist a further polypeptide which is attacked by trypsin but remains unaffected by Tris-washing, and modification of this protein causes rapid D_1^{ox} reduction in trypsinized chloroplasts. Both alternatives are experimentally distinguishable because Tris-washing was found to cause the release of 16, 23 and 33 kDa polypeptides [6,7,26]. Therefore, in the case of mechanism (a), trypsination of Tris-washed inside-out thylakoids should not lead to further modification of the reaction pattern, while in case of mechanism (b) significant changes would be expected to arise.

In order to check these possibilities, laser-pulse induced absorption changes at 320 and 830 nm were measured in Tris inside-out control and Tris inside-out trypsinized thylakoids. The data obtained at pH 7.4 are depicted in Fig. 6. In Tris-washed inside-out control thylakoids, the laser pulse induced 320 nm absorption changes reveal an at least biphasic decay with a prominent 150–200 μs kinetics (sometimes this decay appears to be biphasic with half-life times of the same order of magnitude) and a slower relaxation ($t_{1/2}$ > 100 ms) of minor extent (30–40%). Analogously to normal Tris-washed chloroplasts [26–29], the 150–200 μs kinetics could be interpreted as the Q_A^- reoxidation by P-680$^+$ via an internal back reaction. This idea is supported by the finding that the extent of these kinetics decreases with increasing dark time between the repetitive pulses (data not shown). Furthermore, in the absence of phenyl-*p*-benzoquinone the fast kinetics are often even more pronounced. This might indicate a partial Q_B^- reoxidation by the exogenous acceptor as in the case of normal inside-out thylakoids (see

Fig. 4). Corresponding to the 320 nm measurements, the relaxation of absorption changes at 830 nm, mainly indicating the P-680$^+$ reduction, are also dominated by kinetics in the range of 50–100 μs. These kinetics are somewhat faster than those observed at 320 nm. This phenomenon could be explained by a more complex reaction pattern if one assumes that even repetitive flash excitation at frequencies where D_1^{ox} normally stays highly oxidized in Tris-washed thylakoids, P680$^+$ becomes reduced not only by Q_A^- but also by an additional donor component of unknown chemical nature. Beyond the 50–100 μs kinetics there also appears a minor contribution of a 5–10 μs relaxation that is obviously due to partial D_1^{ox} reduction and, therefore, strongly depends on the dark time between the laser pulses as previously described [11]. Therefore, regardless of the more complex mechanistic details (they are not essential for the conclusions of this paper and will be discussed elsewhere), Tris inside-out thylakoids closely resemble Tris-washed chloroplasts with normal membrane polarity. However, after mild trypsin treatment of Tris inside-out thylakoids, the relaxation kinetics of the absorption changes at 320 and 830 nm become markedly changed. The extent of slow decay kinetics of absorption changes at 320 nm is significantly increased at the expense of relaxation kinetics in the μs range. In contrast to that, at 830 nm the faster 10 μs relaxation dominates in trypsinized Tris inside-out thylakoids, whereas the 50–100 μs kinetics almost completely disappear. These results can be explained consistently by the assumption that mild trypsination stimulates the D_1^{ox} reduction with Q_B^- as electron donor. This effect does not involve the 16, 23 and 33 kDa polypeptides associated with the donor side of PS II, because they are lost to a large extent by Tris-washing [26]. Therefore, the experimental results reported in this study strongly favor mechanism (b) to be responsible for the effect of trypsin, i.e., the accelerated D_1^{ox} reduction does involve the proteolytic modification of a proteinaceous component other than the above-mentioned polypeptides of 16, 23 and 33 kDa. A comparison of the data of Figs. 4 and 6 shows that trypsin treatment at pH 7.4 of normal inside-out and Tris-washed inside-out thylakoids elicits almost the same reaction pattern. A very interesting effect, however, arises if mild trypsination is performed at pH 6.5. In normal inside-out thylakoids neither the oxygen evolving capacity is affected (see Fig. 1) nor the kinetical patterns of the absorption changes at 320 or 830 nm (data not shown). If, however, Tris-washed inside-out thylakoids are trypsinized at pH 6.5 very similar effects are achieved as by treatment at pH 7.4, except for the fast microsecond relaxation kinetics of the 830 nm absorption change being somewhat slower due to pH-dependence of the electron transport from D_1 to P-680$^+$ (see Fig. 5). This provides a complementary line of evidence to support mechanism (b) of trypsination (see Discussion). It should be mentioned that trypsination of Tris-washed inside-out thylakoids does not only stimulate internal D_1^{ox} reduction but exerts additional influence. In the absence of phenyl-*p*-benzoquinone as exogenous electron acceptor, the extent of the absorption changes at 320 and 830 nm becomes reduced under repetitive pulse excitation, whereas in control Tris-washed inside-out thylakoids the initial amplitudes are independent of the presence of phenyl-*p*-benzoquinone within the experimental error of the data. This effect, however, will not be further analyzed in this study.

Discussion

The results presented in this study have to be discussed within the framework of our current knowledge about the structural and functional organization of photosynthetic water cleavage that is schematically summarized by the model in Fig. 7. Extending the previous conclusion of Bricker et al. [6], it is assumed that the functional manganese group for water oxidation (which is likely to be a binuclear cluster) is located within a 34 kDa polypeptide intrinsically bound to the thylakoid membrane. This polypeptide appears to be identical with the 34 kDa unit discovered previously in several mutant phenotypes of *Scenedesmus obliquus* [31]. The surface exposed 16, 23 and 34 kDa polypeptides have a regulatory function only. This idea has been manifested by recent results (some of them came to our knowledge after the completion of this study). It was found that in PS II particles the L-surface (L refers to lumen) exposed lysine-rich 33 kDa unit [32] can be removed (to-

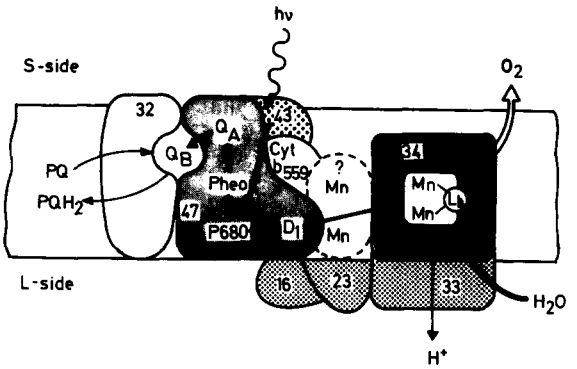

Fig. 7. Simplified scheme for the structural and functional organization of photosynthetic water cleavage (for details see text). L- and S-side refers to lumen side and stroma side, respectively.

gether with the 16 and 23 kDa polypeptides) without any loss of manganese by appropriate $CaCl_2$-treatment [33]. This procedure completely eliminates the oxygen-evolving capacity which can be partly restored by readding the 33 kDa unit [34]. $CaCl_2$ alone was found to substitute the 33 kDa unit for its function in partial restoration of the oxygen-evolving activity as well as for affecting the binding strength of manganese [35]. Accordingly, the L-surface exposed 33 kDa subunit was inferred to stabilize a conformational state that is indispensible for a proper environment of the functional manganese centers [35]. Recently, the isolation of a manganese containing polypeptide by salt-washing has been reported, which was suggested to be the manganese binding subunit of the water-oxidizing enzyme system [36]. However, as the isoelectric point of this protein is identical to that of the L-surface exposed 33 kDa polypeptide, the data of Ono and Inoue [33–35] rather suggest that manganese released from its native binding site might have become attached to the 33 kDa unit. This idea is supported by previous reports on metal center transfer between subunits after harsh treatment of cytochrome oxidase [37].

With regard to the 23 kDa polypeptide, latest data provided evidence for a regulatory function, because this subunit greatly enhances the Cl^--affinity of the native binding site [38]. Therefore, removal of this subunit leads to decrease of the oxygen-evolving capacity due to suboptimal Cl^--binding. The role of the 16 kDa polypeptide has not been unambiguously clarified but many data suggest that it is involved neither directly nor indirectly in the process of water oxidation (but see Ref. 22).

According to Fig. 7, two generally distinct mechanisms can be discussed for the tryptic degradation of the oxygen-evolving capacity and its rather strong pH-dependence. (i) Trypsin modifies only the lysine- and arginine-containing L-surface exposed 16, 23 and 33 kDa regulatory polypeptides, thereby functionally disconnecting the 34 kDa catalytic manganese group from the reaction center. In this case the strong pH-dependence is restricted to a conformationally induced modification of the susceptibility of lysine and/or arginine residues in these three polypeptides. (ii) After only rather slight changes of the surface-exposed polypeptides, trypsin directly attacks the intrinsic 34 kDa catalytic unit so that the pH-effect could be predominantly due to structural changes of this moiety.

An analysis of the effect of mild trypsination and its pH-dependence on the manganese content of inside-out thylakoids might help solve this mechanistic problem. Previous findings led to the conclusion that trypsin treatment of normal chloroplasts leads to deterioration of the oxygen-evolving capacity without manganese detachment [39]. However, in normal chloroplasts trypsin attacks the thylakoid membrane from the S-side (S refers to stroma) of system II so that the effect at the donor side could be due to secondary structural effects and therefore unambiguous conclusions cannot be drawn from these data. Latest experiments show that mild trypsination of PS II particles causes a manganese release which is strongly pH-dependent (Völker, M., Ono, T., Inoue, Y. and Renger, G., unpublished data). This result favors the idea that trypsination modifies not only the regulatory subunits but also changes the microenvironment of the catalytic manganese group. However, the proteolytic digestion pattern is even more complex because, after removal of the 16, 23 and 33 kDa polypeptides by Tris-washing of inside-out thylakoids, which is accompanied by manganese release and complete loss of oxygen-evolving capacity, the D_1^{ox}-reduction kinetics become significantly modified after mild trypsination (see Fig. 6). In normal Tris-washed thylakoids, without

addition of exogeneous electron donors, D_1^{ox} becomes reduced predominantly by Q_B^- and to minor extent by Q_A^- [40]. In Tris-washed inside-out thylakoids the situation is complex because, in the absence of phenyl-*p*-benzoquinone, trypsination decreases the extent of PS II turnover (reflected by the absorption changes at 320 and 830 nm, respectively; data not shown) under repetitive excitation. Therefore, an additional phenyl-*p*-benzoquinone-mediated cyclic electron flow has to be considered but will not be discussed here. However, regardless of these mechanistic details, the data in Fig. 6 indicate that trypsin attacks not only the above-mentioned polypeptides but induces a further modification of system II causing rapid D_1^{ox} reduction. The molecular origin of this effect, which does not reveal the striking pH-dependence of the trypsin-induced deterioration of the oxygen-evolving capacity, remains to be clarified. It appears very unlikely that trypsin changes the microenvironment of the functional redox group of D_1, because the electron transfer kinetics from D_1 to $P\text{-}680^+$ and their pH-dependence (see Fig. 5) are invariant to mild tryptic treatment. Interestingly enough, structural modifications were inferred to be responsible for faster D_1^{ox}-reduction induced by salt depletion of normal Tris-washed chloroplasts [41]. Therefore, it appears reasonable to speculate about a further proteinaceous component which indirectly affects the reaction pattern of system II via structural changes. The present data do not permit definite conclusions about such a component.

Another point which might not be directly related to the trypsination pattern, but on which our attention should be focussed and which is considered in Fig. 7, is the functional heterogeneity of the manganese pool with regard to the magnetic interaction with D_1^{ox} [42]. A comparison with oxidases (laccase, cytochrome oxidase) catalyzing the reverse reaction to water oxidation reveals that the catalytic binuclear transition metal centers are coupled with the electron source via another transition metal group that only mediates the electron transfer (for review, see Ref. 43). An analogous situation could arise for the water-oxidizing enzyme system so that it seems attractive to speculate about another manganese containing redox group which functionally connects the catalytic manganese center via D_1 with the reaction center complex. The present study shows that mild and selective trypsination of inside-out thylakoids provides a valuable tool for analyzing the architecture of the water-oxidizing enzyme system Y.

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