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# Effects of trypsin and bivalent cations on P-680 $^+$-reduction, fluorescence induction and oxygen evolution in Photosystem II membrane fragments from spinach

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**Laser-flash-induced absorption changes at 830 nm, fluorescence-induction curves and the average oxygen yield per flash have been measured in spinach Photosystem II membrane fragments as a function of trypsin treatment and its modification by $CaCl_2$. The following was found. (i) The relative contribution of the nanosecond relaxation to the overall decay kinetics of 830 nm absorption changes reflecting the P-680$^+$-reduction decreases as a function of incubation time with trypsin. Simultaneously, mild treatment at pH = 6.0 markedly increases the extent of 200 μs kinetics that highly revert back to nanosecond kinetics by $CaCl_2$ addition. After harsher trypsin treatment (pH = 7.5) pH-dependent 2–20 μs kinetics appear that cannot be reverted to nanosecond kinetics by $CaCl_2$. (ii) The $CaCl_2$-induced restoration of nanosecond kinetics is mainly due to a $Ca^{2+}$-induced effect rather than to a functional role of $Cl^-$. $Sr^{2+}$ can substantially substitute for $Ca^{2+}$, whereas $Mg^{2+}$, $Mn^{2+}$ and monovalent ions are almost inefficient. (iii) A quantitative correlation between the extent of the nanosecond kinetics and the average oxygen yield per flash was not observed. (iv) If $CaCl_2$ is present in the assay medium for trypsin treatment the samples are markedly protected to proteolytic degradation. This effect mainly refers to the reaction pattern of the acceptor side. Other bivalent cations can substitute $Ca^{2+}$ for its protective function. (v) The $CaCl_2$-induced protection to proteolytic attack is extremely sensitive to a very short trypsin pretreatment that does hardly affect the shape of the fluorescence induction curve. The results are discussed in relation to the functional and structural organization of Photosystem II.**

## Introduction

Photosynthetic water oxidation by visible light takes place in Photosystem II via a four-step univalent redox-reaction sequence initiated by photooxidation of a special chlorophyll *a*, referred to as P-680. Dioxygen formation is assumed to occur at a catalytic site which contains a binuclear manganese center (for a recent discussion, see Ref. 1).

The functional connection between the catalytic binuclear manganese cluster and P-680 implies at least one further redox component, referred to as Z [2]. In samples competent in oxygen evolution, P-680$^+$ reduction exhibits a multiphasic kinetics in the nano- and microsecond range [3–5], whereas the oxidation of the catalytic site was

Abbreviations: PS II, Photosystem II; Chl, chlorophyll; Cyt, cytochrome; Mes, 4-morpholineethanesulphonic acid.

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shown to occur within the range of 30 μs to 1 ms, depending on its redox state $S_i$ [6–8]. If the oxygen-evolving capacity is completely eliminated by selective treatments (e.g., Tris-washing) the P-680$^+$-reduction kinetics markedly slow down: P-680$^+$ becomes reduced either via a pH-dependent electron flow from Z with half-life times of 2–20 μs [9] or via a back reaction ($t_{1/2}$ = 100–200 μs) with the reduced primary plastoquinone acceptor $Q_A^-$ [10,11]. This phenomenon raised the idea that any microsecond kinetics of P-680$^+$-reduction might be indicative of detrimental effects at the water-oxidizing enzyme system Y [12]. In this case the extent of nanosecond kinetics could be used as a measure of the percentage of PS II reaction centers that are functionally coupled with an intact catalytic site for water oxidation [12,13]. However, experiments with ADRY-agents [14] indicated that the extent of a 35 μs kinetics of 690 nm absorption changes depends upon the flash number of repetitive flash groups [15]. Therefore at least part of the microsecond kinetics are related to functionally intact systems Y. Furthermore, based on these data the P-680$^+$ reduction kinetics were inferred to depend on the redox state $S_i$ of the water-oxidizing enzyme system Y [15]. Later much more refined measurements confirmed this basic conclusion [13].

In order to study possible correlations between P-680$^+$-reduction kinetics and the structural and functional integrity of the PS II donor side, experiments have to be performed in properly modified PS II membrane fragments. Mild trypsin treatment at pH = 6.5 leads to degradation of the surface exposed 18 kDa, 23 kDa and 33 kDa polypeptides [16] which act as regulatory subunits (decrease of $Cl^-$ requirement, $Ca^{2+}$ trapping and stabilizing of functional manganese) of the water-oxidizing enzyme system Y (for a recent review, see Ref. 17). Likewise, integral membrane proteins of the PS II reaction center and its associated core antenna (CP47, CP43, D1, D2 and Cyt *b* 559) are attacked by trypsin [16,18]. On the other hand, oxygen evolution with $K_3[Fe(CN)_6]$ as exogeneous electron acceptor remains highly active in trypsinized PS II membrane fragments [16] and inside-out vesicles [19]. Furthermore, under comparable conditions (trypsination at pH = 6.0), the low temperature EPR signals due to light-induced $S_2$ formation (multiline and $g = 4.1$ signal) and of donor D (signal $II_s$) are almost unaffected; whereas, the high potential Cyt *b*-559 is transformed into the low potential species [20]. Accordingly, limited proteolysis of PS II membrane fragments appears to be a proper tool for analyzing the functional and structural topography of PS II. Here we report the effect of trypsin treatment on 830 nm absorption changes that reflect the turnover of P-680 on oxygen evolution and on fluorescence induction. Furthermore, our attention was focused on the effect of $Ca^{2+}$ as an essential cofactor for the function of system Y [21–23] because recent experiments revealed a marked stimulation of the oxygen evolution rate in trypsinized PS II membrane fragments [16].

## Materials and Methods

PS-II particles with a high oxygen-evolving capacity were prepared from market spinach as described in Ref. 24 with the modifications described in Ref. 16, except that the applied Triton X-100/chlorophyll ratio was 20:1. Bovine pancreas trypsin was purchased from Boehringer (Mannheim). For comparitive measurements the proteolytic treatment for the measurements of the oxygen-evolving activity and of absorption changes at 830 nm was carried out under identical conditions, i.e., the PS-II particles (50 μg Chl/ml) were incubated in darkness with trypsin (trypsin/Chl = 2:1) at 20°C in the oxygen-measuring cuvette and after a certain time either illuminated directly for detecting the oxygen yield or transferred to the cuvette of the flash photometer for measuring absorption changes. In all other experiments trypsination was performed in the sample cuvette. In either experiment the dark time between the flashes was 600 ms and the samples were illuminated with the same number of flashes. The flash induced $O_2$-yield was measured as described earlier [14].

The measurements of the 830 nm absorption changes with nanosecond time resolution were performed with a single-beam flash photometer similar to the one described in Ref. 3. The measuring light was provided by a laser diode (TXF 8300 AEG-Telefunken, $\lambda = 834$ nm). By means of a microscope objective lens, the beam was focussed

through the cuvette (4 cm pathlength) onto a 1 mm aperture, which was located in front of the photodetector (Photodiode preamplifier module RCA C 30952 E). In order to suppress a flash-induced fluorescence artefact, the distance between the cuvette and the aperture was approx. 1 m.

The photodiode was protected against fluorescence by a 830 nm interference filter and coupled via a 50 MHz amplifier (Pacific 2 A 50) to a Tektronix 7912 digitizer. 128 signals were averaged and stored on floppy disks.

In order to make sure that the total initial amplitude of the absorption change in the nanosecond time range could be detected with our time resolution an additional check was performed. $NH_2OH$ (3 mM, 6 min dark incubation) was added to the sample after each experiment that leads to retardation of the relaxation kinetics at 830 nm, due to an inhibition of the water-oxidizing enzyme. The total amplitudes of the 830 nm absorption changes in the presence of $NH_2OH$ are not limited by time resolution of our equipment. Accordingly, the extent of the nanosecond component related to the corresponding control value was calculated in two different ways: (1) the portion of the amplitude that decay within 1 μs related to the total amplitude at $t = 0$; and (2) the portion of the amplitude that decays within 1 μs related to the total amplitude in $NH_2OH$-treated samples at $t = 0$.

In experiments with microsecond time resolution, the photodetector was coupled to a 1 MHz amplifier (Tektronix AM 502) and the signals were transferred to a Nicolet 1170 averager.

Photosynthesis was excited by non saturating pulses from a Q-switched frequency-doubled NdYAG-laser (Spektrum GmbH, Berlin, $\lambda = 534$ nm; duration, 7 ns).

All experiments were carried out with a chlorophyll concentration of 50 μg/ml in a medium containing 10 mM NaCl and 20 mM Mes/NaOH (pH 6.0) with 1 mM $K_3[Fe(CN)_6]$ as an artificial electron acceptor. Other additions, as indicated in the figure legends. Fluorescence was monitored as described in Ref. 25. For these measurements the chlorophyll concentration was 5 μM.

## Results

The P-680 turnover can be monitored by absorption changes peaking around 820 nm [3,11]. Typical traces of absorption changes at 830 nm induced by repetitive flashes in spinach PS II membrane fragments are depicted in Fig. 1. The relaxation kinetics that reflect the reduction of $P\text{-}680^+$ exhibits a multiphasic pattern. In oxygen-evolving control samples at pH = 6.0 the decay is dominated by nanosecond kinetics contributing approx. 65–70% to the overall relaxation. Addition of 10 mM $CaCl_2$ further increases the relative extent of the nanosecond kinetics up to 80%. Destruction of the oxygen-evolving capacity by incubation with 3 mM $NH_2OH$ for 6 min is accompanied with complete elimination of the nanosecond kinetics. In these samples $P\text{-}680^+$ ex-

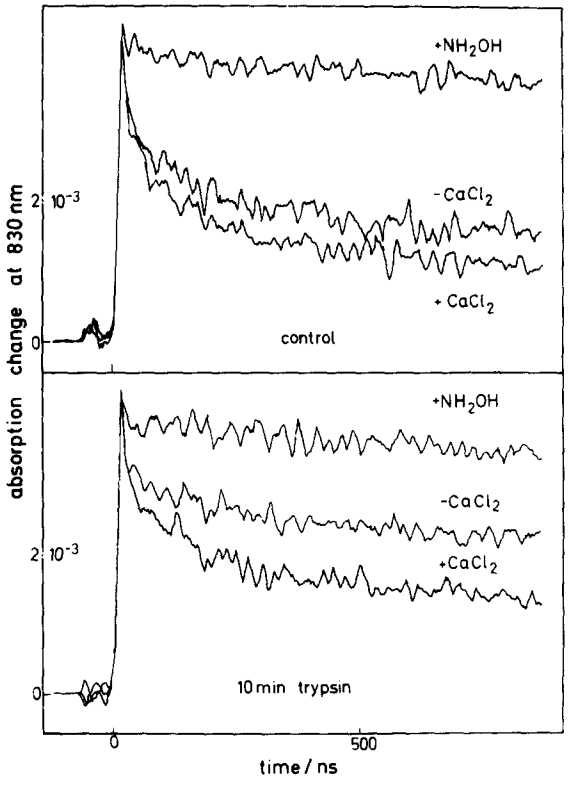


Fig. 1. Absorption changes at 830 nm induced by repetitive laser flashes in normal and trypsinized PS II membrane fragments at pH = 6.0 in the absence and after addition of 10 mM $CaCl_2$. Experimental conditions as described in Materials and Methods.

hibits half-life times in the microsecond range that depend upon illumination conditions [26]. The invariance of the initial amplitude to $NH_2OH$ treatment indicates that the detection of the nanosecond kinetics of P-680$^+$ reduction is not limited by the time resolution of our equipment. After incubation of PS II membrane fragments with trypsin at pH = 6.0 the extent of microsecond relaxation kinetics increases (up to more than 50%) at the expense of nanosecond components. This effect appears to be interesting in the light of recent findings, indicating that oxygen evolution was hardly affected by mild trypsin treatment at pH = 6.0 in PS II-membrane fragments [16] and inside-out vesicles [19], whereas the polypeptide pattern became modified [16]. The relaxation kinetics of 830 nm absorption changes was analyzed as a function of trypsin treatment. A thorough analysis reveals that at least three different kinetics with half-life times of the order of 10 μs, 200 μs and more than 1 ms (these kinetics will be referred to as 10-μs, 200-μs and slow components, respectively) contribute to the decay in the micro- to millisecond time domain (data not shown).

Fig. 2 shows the extent of the different kinetics related to the overall relaxation of 830 nm absorption changes as a function of incubation time with trypsin at pH = 6.0. The data reveal a marked decrease of the relative extent of nanosecond kinetics (for the sake of simplicity a separation into different nanosecond components was not performed) and a concomitant increase of contributions in the micro- to millisecond range. Recently, it was found that trypsin treatment at pH = 7.5 highly reduces oxygen evolution and elicits the pH-dependent 2–20 μs kinetics [17,26] which are characteristic for the electron transfer from Z to P-680$^+$ after destruction of the water-oxidizing enzyme system [9]. Accordingly, it seemed worthwhile to study the pattern of relaxation kinetics as a function of trypsin treatment at pH = 7.5. The results of Fig. 3 reveal an interesting phenomenon. At the beginning, the 200-μs component (also the slow component, data not shown) sharply rises and subsequently steeply declines, whereas the 10-μs component continues to increase with increasing incubation time. The most simple explanation for the appearance of 200-μs

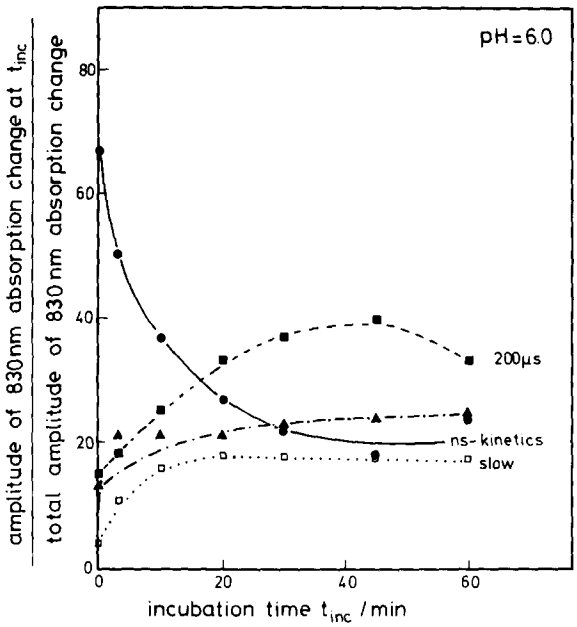


Fig. 2. Relative extent of different kinetics in the overall decay of 830 nm absorption changes in PS II membrane fragments as a function of incubation time with trypsin at pH = 6.0. Experimental conditions as described in Materials and Methods.

kinetics is the assumption that after elimination of the function of water to act as an electron donor $Z^{ox}$ remains oxidized under repetitive flash excitation and therefore a back reaction arises between P-680$^+$ and $Q_A^-$. This interpretation would imply that progressing trypsination accelerates the regeneration of $Z^{ox}$ (as shown for inside-out vesicles, see Ref. 19) so that under the same excitation conditions the extent of the 10-μs component increases. The P-680$^+$-reduction pattern in mildly trypsinized (pH = 6.0) PS II membrane fragments is markedly affected by addition of $CaCl_2$. In respect to the origin of the $CaCl_2$ reversible kinetics arising due to mild trypsin treatment, an interesting finding should be mentioned. The extent of the 200-μs relaxation kinetics is not affected by exogeneous PS II donors (data not shown) that are known to feed electrons efficiently to $Z^{ox}$, if the water-oxidizing enzyme system Y is destroyed (e.g., by Tris-washing), but do not compete with dioxygen evolution in functionally competent samples. This result indicates that the shielding effect exerted by polypeptides

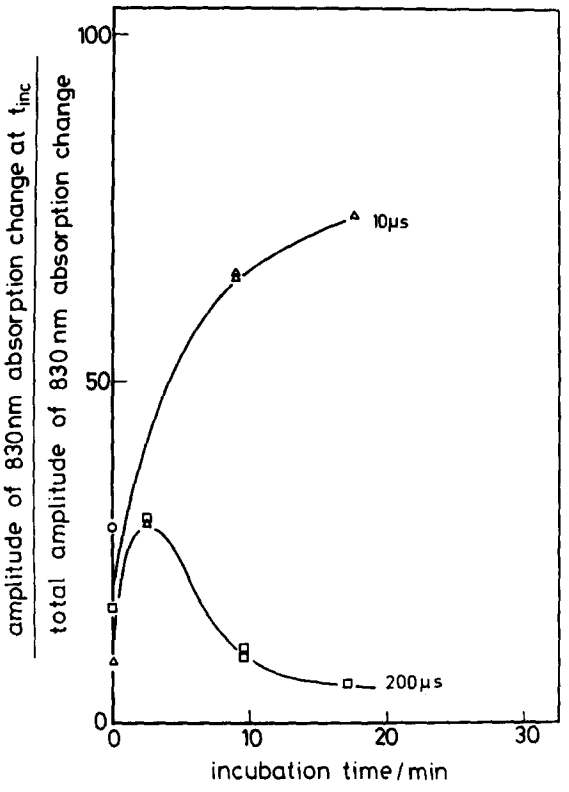


Fig. 3. Relative extent of the 10-µs and 200-µs kinetics in the overall decay of 830 nm absorption changes in PS II membrane fragments as a function of incubation time with trypsin at pH = 7.5. Experimental conditions as described in Materials and Methods.

which are probably related to the water-oxidizing enzyme system Y is not eliminated by the mild trypsin treatment. On the other hand, the pattern of P-680$^+$ reduction becomes significantly affected. Therefore, it appears reasonable to assume that a mild proteolytic attack causes structural changes which modify the functional connection between reaction center and water-oxidizing enzyme system Y without degrading the functional capacity of the latter operational unit. In Fig. 1 the extent of the nanosecond kinetics was shown to become increased by addition of $CaCl_2$. This effect is pronounced in PS-II membrane fragments trypsinized at pH = 6.0. Therefore, it was interesting to analyze the $CaCl_2$-induced transformation of the relaxation pattern. Fig. 4 depicts the relative extent of ns, 10-µs and 200-µs components as a function of incubation time with trypsin at pH = 6.0 and of its modification by addition of 10 mM $CaCl_2$ after the indicated time of proteolysis. The data reveal two characteristics: (a) $CaCl_2$-addition partly restores the contributions of nanosecond kinetics and (b) the 200-µs component is markedly reduced after $CaCl_2$ addition, whereas the 10-µs kinetics remains almost unaffected.

The partial restoration of the nanosecond kinetics by $CaCl_2$ could be related to specific effects of $Ca^{2+}$ and/or $Cl^-$ [21–23,27–29] or simply caused by structural effects due to nonspecific electrostatic interactions that are changed by salt addition. In order to test these alternatives experiments with different salts were performed in trypsinized PS II membrane fragments. A relative simple method was applied for the determination of the restoration degree. The results of Fig. 1 show that the extent of nanosecond kinetics can be easily determined as the difference of the am-

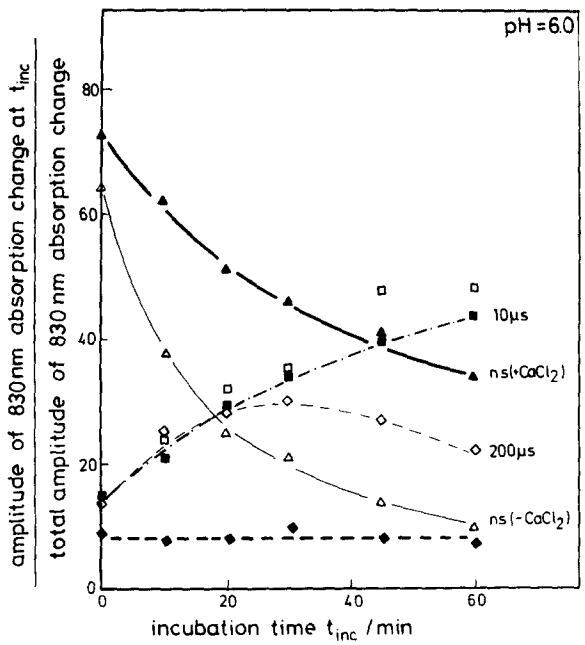


Fig. 4. Relative extent of the ns, 10-µs and 200-µs kinetics in the overall decay of 830 nm absorption changes in PS II membrane fragments as a function of incubation time with trypsin at pH = 6.0 in the absence and after addition of 10 mM $CaCl_2$. Experimental conditions as described in Materials and Methods. Open symbols, experiments without $CaCl_2$; closed symbols, with $CaCl_2$.

plitudes at 1 μs, measured in the presence of 3 mM $NH_2OH$ and its absence, respectively, i.e.

$$\Delta A^{ns}_{830} = \Delta A^{1\mu s}_{830}(+NH_2OH) - \Delta A^{1\mu s}_{830}(-NH_2OH)$$

This relation can be applied only if the 830 nm absorption changes remain invariant (in extent and kinetics up to 1 μs) to different salt additions. This was shown to be practically the case for all salts tested (data not shown). Taking into account $\Delta A^{1\mu s}_{830}(+NH_2OH)$ = constant, the reconstitution factor related to the $CaCl_2$ effect can be expressed by Eqn. 1:

$$\Delta R^{ns}_{salt} = \left( \frac{\Delta A^{1\mu s}_{830}(\text{control}) - \Delta A^{1\mu s}_{830}(\text{salt})}{\Delta A^{1\mu s}_{830}(\text{control}) - \Delta A^{1\mu s}_{830}(10\text{ mM } CaCl_2)} \right)_{\text{trypsin}} \quad (1)$$

where $\Delta R^{ns}_{salt}$ represents the normalized reconstitution factor of the extent of the nanosecond kinetics in PS II membrane fragments trypsinized at pH = 6.0. 10 mM $CaCl_2$ was used as reference, because at this concentration the restoration effect completely saturates [30].

The data obtained for different salts are summarized in Table I in terms of percentage reconstitution, i.e. $100 \times \Delta R^{ns}_{salt}$. The results clearly demonstrate that the restoration of the nanosecond kinetics is dominated by $Ca^{2+}$ and that $Cl^-$ plays only a marginal role. $Ca^{2+}$ can be substituted to a marked extent by $Sr^{2+}$ and partly by $Ba^{2+}$, whereas $Mg^{2+}$, $Mn^{2+}$ and monovalent cations are almost inefficient. It should be mentioned that $Ba^{2+}$ causes additionally a time-dependent inhibition. Therefore the data of Table I for $Ba^{2+}$ refer only to the initial activity. The degree of the conversion of nanosecond kinetics of P-680$^+$-reduction in PS II membrane fragments into decay components in the microsecond range by mild trypsin treatment at pH = 6.0 as well as the extent of restoration by 10 mM $CaCl_2$ varied quantitatively for different preparations. The same qualitative behaviour, however, was observed in all samples that were used in this study. The above-mentioned results support the idea that a specific structural modification affects the reduction kinetics of P-680$^+$. Now the question arises about the functional site of the trypsin-induced effect and its partial reversal by $Ca^{2+}$. Fig. 5 depicts the absorption changes induced by a flash

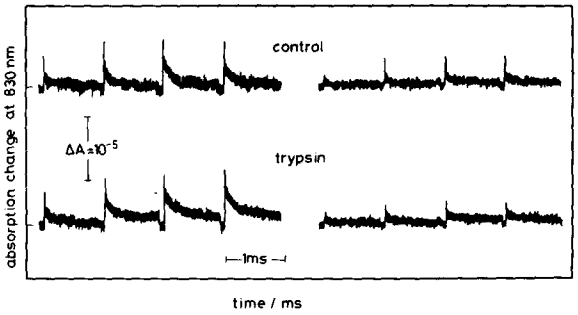


Fig. 5. Absorption changes at 830 nm induced by a train of four flashes in dark-adapted normal and trypsinized PS II membrane fragments at pH = 6.0 in the absence (left side) and presence (right side) of 10 mM $CaCl_2$. Experimental conditions as described in Materials and Methods.

TABLE I

| Salt added | $\frac{\Delta A^{1\mu s}_{830}(\text{control}) - \Delta A^{1\mu s}_{830}(\text{salt})}{\Delta A^{1\mu s}_{830}(\text{control}) - \Delta A^{1\mu s}_{830}(10\text{ mM } CaCl_2)} \cdot 100\%$ |
|---|---|
| 10 mM $CaCl_2$ | 100 |
| 10 mM $Ca(NO_3)_2$ | 92 |
| 10 mM $Ca(OAc)_2$ | 89 |
| 10 mM $Ca(OAc)_2$ (assay medium − $Cl^-$) | 74 |
| 10 mM $SrCl_2$ | 85 |
| 10 mM $BaCl_2$* | 43 |
| 10 mM $MgCl_2$ | 8 |
| 10 mM $MnCl_2$ | 29 |
| 10 mM NaCl | 0 |
| 100 mM NaCl | 15 |
| 10 mM KCl | 0 |

train in dark-adapted PS II membrane fragments. The data indicate a progressive increase of the slow kinetics during the flash train (the steady state is reached only after 20–30 flashes, data not shown). This pattern is not indicative for a selective blockage of $Z^{ox}$-reduction at a specific redox state $S_i$ (see Discussion). In order to test a possible correlation between the extent of the nanosecond kinetics and the oxygen evolving capacity, the average oxygen yield per flash was measured under comparable experimental conditions (see Materials and Methods). In most of our experiments oxygen was found to be less sensitive than the nanosecond kinetics to mild trypsin at pH = 6.0, as is shown in Fig. 6. Unfortunately, we observed for different preparations marked deviations from the pattern depicted in Fig. 6. Therefore unambiguous conclusions cannot be drawn at this point. However, the opposite effect, i.e., persistence of nanosecond kinetics to specific treatments that highly suppress oxygen evolution, was recently well established [31]. Therefore, the relative extent of the nanosecond kinetics of P-680$^+$ reduction cannot be used as an unambiguous quantitative measure for the percentage of functionally competent water-oxidizing enzyme systems. So far we have analyzed the modification of the P-680$^+$-reduction pattern by mild trypsin treatment at pH = 6.0 and the $Ca^{2+}$-induced partial restoration. Recently, it was shown that $CaCl_2$ in the assay medium affects also the susceptibility to trypsin of herbicide binding and the *p*-benzoquinone-mediated electron transport in PS II membrane fragments [32]. This protective effect to tryptic attack was further investigated by measurements of room temperature fluorescence induction curves in PS-II membrane fragments that were trypsinized at pH = 7.5 in the absence or presence of 10 mM $CaCl_2$. Fig. 7 shows the effect of increasing incubation time with trypsin. After a few minutes trypsination in the absence of $CaCl_2$ interruption takes place of electron transfer from $Q_A^-$ to $Q_B$. This effect causes a fast fluorescence rise. The retardation of the subsequent slower-rising part probably reflects two effects: a fraction of the PS-II centers could remain connected with the total plastoquinone pool, and in addition the donor side activity degrades too. Further, the decrease of the maximum fluorescence reflects a trypsin-induced quenching, probably by effects of trypsin on the light-harvesting complex [33–35]. A markedly different pattern is observed, if trypsin treatment is performed in the presence of $CaCl_2$. Under these conditions, the acceptor side is highly protected against tryptic attack as shown by the invariance of the fast-rising part of variable fluorescence. This result is in perfect agreement with corresponding lines of evidence from herbicide binding studies and measurements of *p*-BQ-mediated electron transfer [32].

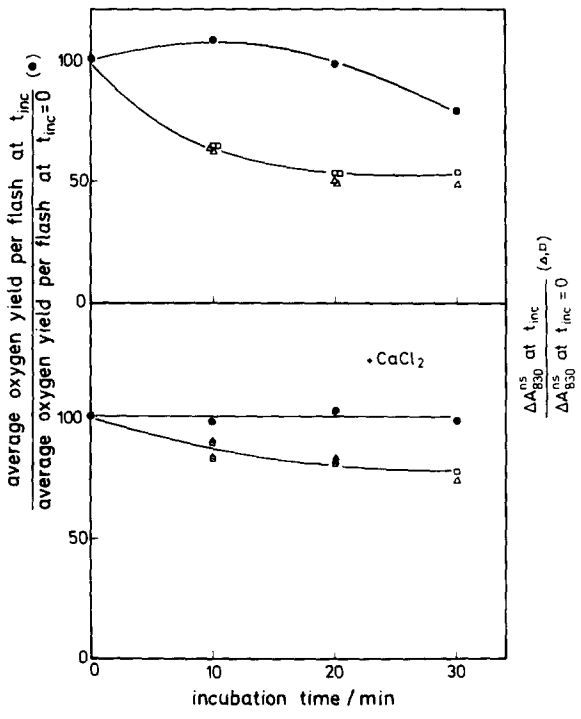


Fig. 6. Average oxygen yield per flash and extent of the nanosecond kinetics in the overall decay of 830 nm absorption changes in PS II membrane fragments as a function of incubation time with trypsin at pH = 6.0 in the absence (top) and after additions of 10 mM $CaCl_2$ (bottom). Experimental conditions as described in Materials and Methods.

In PS II membrane fragments trypsinized at pH = 7.5 in the absence of $CaCl_2$ the shape of the fluorescence induction curve becomes only slightly changed after addition of 3 mM $NH_2OH$ (data not shown). This result indicates that the trypsin-induced blockage at the acceptor side involves a large fraction of the PS II reaction centers. A markedly different pattern is observed if 10 mM $CaCl_2$ is present during the 10 min trypsin treat-

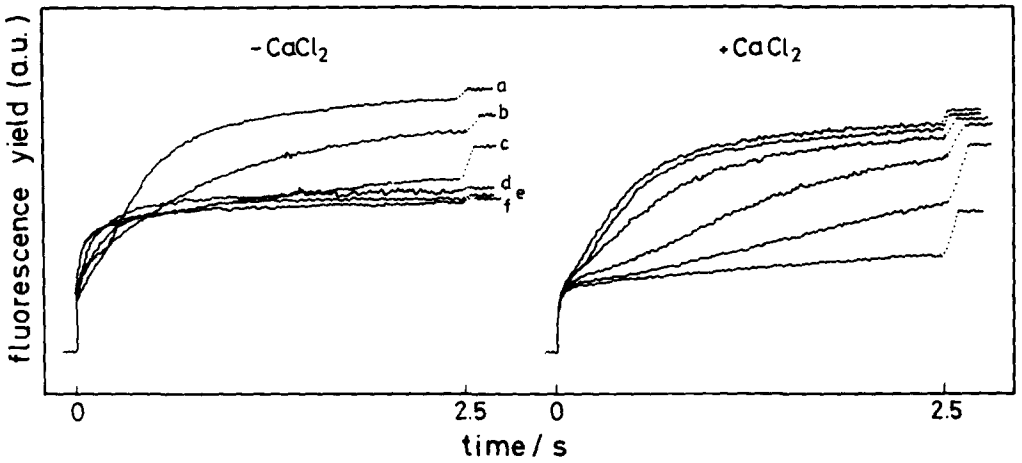


Fig. 7. Fluorescence yield as a function of actinic illumination in normal and trypsinized PS II membrane fragments: (a) control; (b) 1 min; (c) 3 min; (d) 5 min; (e) 10 min and (f) 20 min. PS II membrane fragments were trypsinized at pH = 7.5 in the absence or presence of 10 mM $CaCl_2$. The level indicated at the end of each induction curve was taken after 7 s. Experimental conditions as described in Materials and Methods.

ment. In this case, the acceptor side is highly protected against tryptic attack, whereas the donor-side capacity appears to be diminished due to degradation of the water-oxidizing enzyme system. Addition of 3 mM $NH_2OH$ almost completely restores the inducation curve of the control samples (data not shown). Accordingly, in the presence of $CaCl_2$ the donor side is less resistant to tryptic attack than the acceptor side.

Fluorescence measurements also show [30] that the protective function of $Ca^{2+}$ regarding further

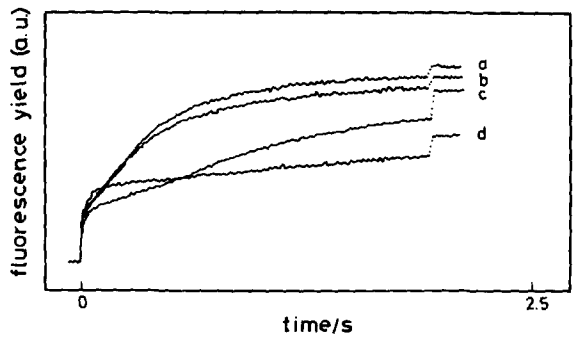


Fig. 8. Fluorescence yield as a function of actinic illumination time in PS II membrane fragments: (a) control; (b) trypsinized for 20 s at pH = 7.5 in the absence of $CaCl_2$; (c) trypsinized for 10 min in the presence of 10 mM $CaCl_2$; (d) trypsinized for 20 s, then addition of 10 mM $CaCl_2$ and further trypsination up to 10 min. Other experimental conditions as described in Material and Methods.

proteolytic degradation disappears after trypsin treatment of PS II membrane fragments. Two questions are of mechanistic relevance. (a) Does the loss of $Ca^{2+}$ protection after trypsin treatment kinetically coincide with the proteolytic degradation of PS II? (b) Does the protective function exhibit the same specificity as the partial restoration effect of the nanosecond kinetics?

The experimental data depicted in Fig. 8 show that a trypsin treatment of only 20 s is sufficient for preventing the protective action of added $CaCl_2$. On the other hand, the 20 s trypsination itself hardly affects the fluorescence curve. This interesting finding indicates that the potency of $Ca^{2+}$ to act as protectant is lost after a rather short trypsin treatment which does not affect the general pattern of PS II electron transport. Specific surface modifications are assumed to be responsible for this phenomenon. Referring to the specificity of the $Ca^{2+}$-induced protection to trypsin experiments were performed with different salts. The protective effect was found to be much less specific than the restoration of the nanosecond kinetics. All bivalent ions tested exhibit similar effects.

## Discussion

The present study shows that trypsin treatment at pH = 6.0 markedly affects the P-680$^+$ reduction

by transforming part of the nanosecond kinetics into markedly slower kinetics. Taking into account the effect of trypsin on the polypeptide pattern [16,19] the data reflect a structural modification of the PSII donor side leading to a change of the electron-transport pattern. For the 200-μs kinetics this modification can be functionally compensated to a significant extent by the specific action of $Ca^{2+}$ salts. This phenomenon implies different questions of mechanistic relevance for the functional and structural organization of the PS II donor side. (1) What is the origin of the trypsin-induced 200-μs reduction kinetics of P-$680^+$? (2) What is the nature of the $Ca^{2+}$-binding site? (3) What is the functional role of $Ca^{2+}$?

Two alternative interpretations can be proposed for the trypsin-induced 200-μs back reaction: (a) blockage of electron transport from the water-oxidizing enzyme system Y to $Z^{ox}$ giving rise to a back reaction between P-$680^+$ and $Q_A^-$, or (b) retardation of the electron-transfer kinetics from Z to P-$680^+$. The former proposal could be in line with recent reports on NaCl-washed samples. Measurements of ultraviolet absorption changes (320 nm) and of the average oxygen yield per flash in inside-out vesicles indicated that after removal of the 23 kDa protein a back reaction between P-$680^+$ and $Q_A^-$ probably arises under repetitive flash excitation at the expense of the oxygen-evolving capacity [36]. Based on delayed fluorescence and oxygen-yield measurements in dark-adapted NaCl-washed PS II membrane fragments deprived of their 18 and 23 kDa polypeptides the redox transition $S_3Z^{ox} \rightarrow S_0Z + O_2 + 2H^+$ was inferred to be blocked. The reaction can be restored by $Ca^{2+}$ [37]. Likewise, under comparable conditions a partial loss of oxygen evolution and a concomitant increase of the EPR signal $II_f$ have been observed that can be reversed by $Ca^{2+}$, but not by other cations [22,38]. Therefore, our present data could be analogously explained by a trypsin-induced degradation of the 18 and 23 kDa polypeptides which causes a $Ca^{2+}$ reversible blockage of $Z^{ox}$ reduction by $S_3$. This would lead to a P-$680^+$ $Q_A^-$ back reaction under repetitive flash excitation. Measurements of 320 nm absorption changes (data not shown) are not in contradiction to this idea, but do not provide an unambiguous proof. However, other tests are available. If the appearance of the 200-μs kinetics at the expense of the nanosecond reduction of P-$680^+$ after mild trypsin treatment (pH = 6.0) is exclusively due to blockage of $S_3$ oxidation by $Z^{ox}$, then two effects should be observed: (a) stoichiometric correlation between the average oxygen yield per flash and the extent of the nanosecond kinetics; (b) excitation of dark-adapted trypsinized samples with a flash train should exhibit a rather small extent of the 200-μs kinetics after the first three flashes followed by a marked increase after the 4th flash due to formation of the state $S_3 \| Z^{ox}$P-$680^+$ $Q_A^-$. Both phenomena have not been observed at the expected degree (see Figs. 5 and 6). Therefore, we conclude that trypsin treatment affects the electron-transfer rate not only between the water-oxidizing enzyme system Y and $Z^{ox}$, but also between Z and P-$680^+$. The quantitative correlation of these effects remains to be clarified.

The trypsin-induced functional modification of the PS II donor side appears to be closely related to the site of $Ca^{2+}$ action. It is now well established that the surface-exposed polypeptides of 18, 23 and 33 kDa do not contain the $Ca^{2+}$-binding site (for a recent review, see Ref. 17). Recently, it was shown that after removal of the 23 kDa polypeptide a light-induced conformational change takes place at a not yet identified intrinsic polypeptide that opens the $Ca^{2+}$-binding site to the outer aqueous medium [39]. $Ca^{2+}$ release is assumed to cause blockage of oxygen evolution. Addition of sufficient exogenous $Ca^{2+}$ saturates the binding site and restores oxygen evolution via an unknown mechanism. Based on thermoluminescence and oxygen-yield measurements in dark-adapted PS II membrane fragments, $Ca^{2+}$ was inferred to be required for the functional integrity of system Y through an all-or-none-type mechanism [40]. Furthermore, it is interesting to note that after depletion of the surface-exposed polypeptides the $Ca^{2+}$-induced restoration of oxygen evolution exhibits a marked heterogeneity [40]. A similar heterogeneity of the $Ca^{2+}$ effect has been also observed in PS II membrane fragments deprived of their 18 kDa and 23 kDa polyeptpides by NaCl washing. The existence of a high affinity (50–100 μM) and a low affinity (1–2 mM) binding site was reported [41]. A quantita-

tive analysis of the restoration effect of P-680$^+$-reduction kinetics reported here as a function of $CaCl_2$ concentration led to the conclusion that the $Ca^{2+}$ effect is a cooperative effect implying at least two binding sites with affinities of 60 μM and 1.3 mM [30]. These values are in close correspondence with the above-mentioned data [41] and latest findings in NaCl-washed wheat PS II-membrane fragments [42]. This suggests that the effect induced by mild trypsin treatment (pH = 6.0) is caused predominantly by microenvironmental changes of the same $Ca^{2+}$-binding site(s) that is (are) also affected by NaCl washing. Therefore, removal (NaCl washing) or desintegration (trypsin, pH = 6.0) of the surface-exposed polypeptides markedly affects the binding of $Ca^{2+}$ that is functionally relevant to water oxidation. However, it remains to be clarified whether the regulatory function of $Ca^{2+}$ implies only one binding site per PS II or two cooperatively interacting binding sites. As $Ca^{2+}$ cannot act as a redox active group its function is assumed to be a tuning of the reaction coordinates at the donor site by structural 'calibration' of the polypeptide matrix and/or electrostatic affects. Our data confirm that the effect of $Ca^{2+}$ is rather specific [22]. The mechanistic implications of this specificity have to be identified in future work. It is interesting to note that after harsher trypsin treatment (pH = 7.5), a pH-dependent 2–20 μs kinetics emerges for the P-680$^+$ reduction which cannot be reversed by $Ca^{2+}$. In this case the water-oxidizing enzyme system becomes irreversibly destroyed [16].

Beyond the $Ca^{2+}$ restoration of functional defects at the donor side due to $Ca^{2+}$ release after mild trypsin treatment (pH = 6.0) a marked protection effect against proteolytic degradation of the acceptor side is observed, if $Ca^{2+}$ is present in the assay medium. This effect is very likely related to a tight membrane appression which hampers trypsin attack. The implications of this protection shall not be discussed extensively in this study.

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