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Effects of trypsin upon EPR signals arising from components of the donor side of Photosystem II

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EPR signals from components functioning on the electron donor side of Photosystem II (PS II) have been monitored in PS II membranes isolated from spinach chloroplasts after treatment with trypsin at pH 7.5 and pH 6.0. The following information has been obtained. (1) The multiline manganese signal, the $g = 4.1$ signal and Signal II_{slow} are lost with trypsin treatment at pH 7.5, but not at pH 6.0. (2) At pH 7.5 the multiline S_2 signal and the $g = 4.1$ signal are lost with approximately the same dependency on the incubation time with trypsin. At pH 6.0 trypsin treatment is known to block electron transfer between Q_A and Q_B (the first and the second quinone electron acceptors, respectively) allowing only a single turnover to occur. Under these conditions both the $g = 4.1$ signal and the multiline signal are induced by illumination at 200 K and their amplitudes are almost the same as in untreated samples. These results are interpreted as indicating that the $g = 4.1$ signal arises from a side path donor or from S_2 itself rather than a carrier functioning between the S states and the reaction center as previously suggested. (3) Cytochrome *b*-559 is converted to its oxidized low-potential form by trypsin treatment at both values of pH. At pH 6.0 the S-state turnover still occurs indicating that the presence of reduced high-potential cytochrome *b*-559 is not necessary for this process.

Introduction

Many structural and functional details of photosynthetic water oxidation to molecular oxygen are unresolved questions (for a recent review, see Ref. 1). One way to attack this problem is to modify specifically the biological apparatus and to analyze the effects on the reaction pattern. It has recently been shown that the donor side of Photosystem II (PS II) exhibits a remarkable pH dependence in its susceptibility to proteolysis by trypsin [2,3]. In preparations where the donor side was exposed to added trypsin the oxygen-evolving capacity was only marginally affected at pH 6.0, but was destroyed at pH 7.5 [2,3]. The pH dependence of the proteolytic activity of trypsin could not account for this effect [2,3]. Accordingly, it was proposed that a structural change took place due to a deprotonation which exposed the O_2-evolving enzyme to tryptic attack at pH 7.5 [2,3].

Abbreviations: Cyt *b*-559, cytochrome *b*-559; Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; Q_A, the first quinone acting as an electron acceptor; Q_B, the second quinone acting as an electron acceptor; S_2, a charge-storage state of the oxygen-evolving enzyme.

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This may also be associated with the observations that EDTA and cyanide inhibit O_2 evolution only at high pH (see Ref. 4).

Low-temperature EPR has recently become an important method for studying PS II photochemistry (reviewed in Refs. 5 and 6). Several EPR signals attributed to donor side components have been reported. In this work we have monitored these EPR signals during trypsin treatment. The results confirm that the action of trypsin is markedly pH dependent and also provide new information on the roles of the components giving rise to the EPR signals.

Materials and Methods

Oxygen-evolving PS-II-enriched membranes were prepared from spinach according to the procedure described in Ref. 7 with modifications as in Ref. 8. The thylakoid fragments, prepared freshly, were treated with 5 mg/ml trypsin in the dark at 20°C at a chlorophyll concentration of 5 mg/ml in an incubation medium containing 10 mM NaCl, 5 mM $MgCl_2$ and either 20 mM Hepes (pH 7.5) or 20 mM Mes (pH = 6.0). Incubation at this high concentration was used because incubations using low chlorophyll concentrations led to decreases in the amplitude of the multiline EPR signal and the $g = 4$ signal in control samples. After a specific incubation time the samples were transferred to EPR-tubes, mixed with EDTA to a concentration of 1 mM and frozen to liquid nitrogen temperature. The tryptic digestion was stopped by this freezing step.

EPR-measurements were made using a Bruker ER-200-X-band spectrometer fitted with an Oxford Instrument liquid He temperature cryostat and control system.

Samples were illuminated using an 800 W projector at 200 K for 4 min in solid CO_2/ethanol bath or at 77 K for 20 min in liquid nitrogen. Other additions were as described in the figure legends.

In order to check the effect of extensive trypsination at pH = 7.5 on the reaction center activity, the absorption changes at 830 nm were measured reflecting the turnover of P-680. Experiments were performed as described in Ref. 2 (but with markedly improved sensitivity and time resolution). It was found that the number of intact PS II reaction centers remained almost unaffected, even after 1 h of trypsin treatment at pH = 7.5 (data not shown).

Results and Discussion

The S_2 multiline signal

A multiline EPR signal arising from a mixed valence manganese cluster and attributed to S_2 [9–12] is observed in O_2-evolving PS II particles when they are illuminated at 200 K [11]. This signal is present in untreated samples at both pH 6.0 and pH 7.5 (Fig. 1). The multiline signals observed at each pH value are spectrally similar and have similar amplitudes as noted previously [13]. Trypsin treatment for 30 min at pH 6.0 resulted in little or no change in the amplitude of the multiline signal, while the same treatment at pH 7.5 resulted in an almost complete loss of the signal.

It has been demonstrated earlier that removal of the extrinsic polypeptides with molecular weights of 17, 23 and 33 kDa results in the loss of the multiline EPR signal [14–16]. It was concluded that S-state turnover was blocked under these conditions. However, it has been clearly shown that S-state turnover does take place in the absence of the extrinsic polypeptides [17–20]. It was pointed out that the loss of the multiline signal does not necessarily reflect the loss of the S_2 state, but only the loss of the complex environment required for its manifestation as the multiline signal [21]. The presence of the multiline signal after trypsin treatment at pH 6.0 indicates that this complex environment is unaltered by this treatment.

It was shown earlier that the 17, 23 and 33 kDa extrinsic polypeptides were all at least partially digested by trypsin even at pH 6.0. A 15 kDa fragment of the 33 kDa polypeptide remained bound to the membrane allowing unaltered function of the O_2-evolving enzyme [3]. Chloride can replace the 17 and 23 kDa polypeptides under some conditions and restores the ability to observe the multiline EPR signal [15]. Since in the present work 15 mM chloride was present in the reaction mixture, we have not determined whether functional fragments of the 17 and 23 kDa poly-

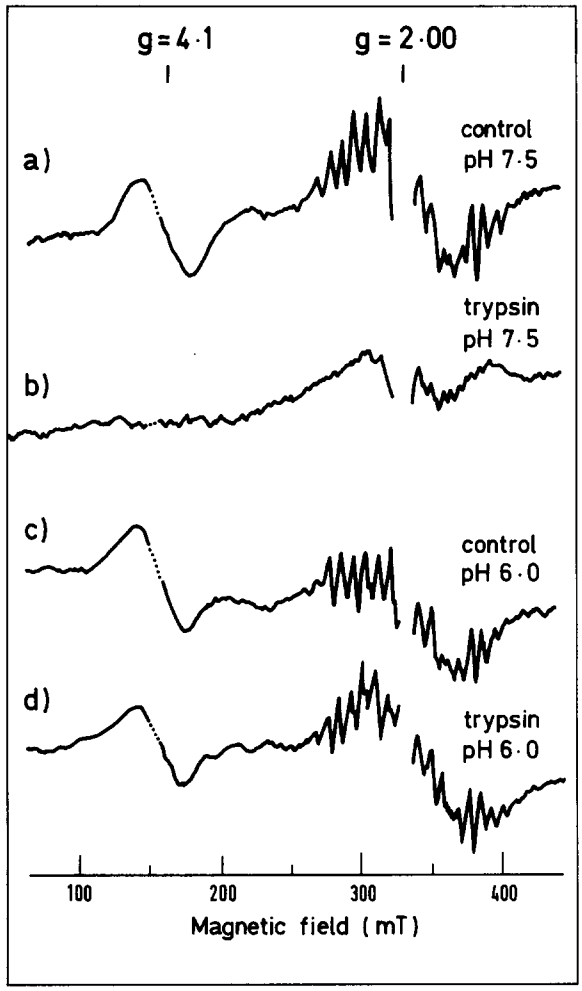


Fig. 1. Light-induced EPR signals in PS II particles and the effect of trypsin. Samples of PS II particles (5 mg Chl/ml) were illuminated for 4 min at 200 K. The spectra shown are 200 K illuminated-minus-dark spectra and are the average of four scans. EPR instrument settings were as follows: temperature, 8 K, microwave power, 8 dB down from 200 mW; modulation amplitude, 20 gauss (a) Untreated control at pH 7.5, (b) trypsin treated for 30 min (pH 7.5); (c) untreated control at pH 6.0; (d) trypsin treated at pH 6.0.

peptides remain associated with the O_2-evolving system or if their functional role is replaced by chloride. In previous work [3] the presence of a functional fragment of the 23 kDa after trypsin treatment was shown to be unlikely.

The $g = 4.1$ signal

An EPR signal associated with the donor side of PS II has been reported at $g = 4.1$ [12,22]. The signal was attributed to an oxidized electron carrier and it was proposed that this component might function in electron transport between the S states and the reaction center [12,22].

Fig. 1 shows that the $g = 4.1$ signal is formed in addition to the multiline signal by illumination at 200 K. The signal is spectrally similar at both pH values. Trypsin digestion at pH 6.0 has little effect on the $g = 4.1$ signal while the same treatment at pH 7.5 results in loss of the signal.

If the $g = 4.1$ signal arises from a component closer to the reaction center than the S state, upon trypsin digestion the multiline might be expected to decay more rapidly than the $g = 4.1$ signal. However, Fig. 2 shows a time-course experiment which demonstrates that the $g = 4.1$ signal and the multiline S_2 signal are lost during trypsin treatment approximately simultaneously.

In other experiments more rapid loss of both signals was observed but the relative rate of the loss of the two signals was the same.

The attribution of the $g = 4.1$ signal to a component functioning between the S states and the reaction center was partially based on the observa-

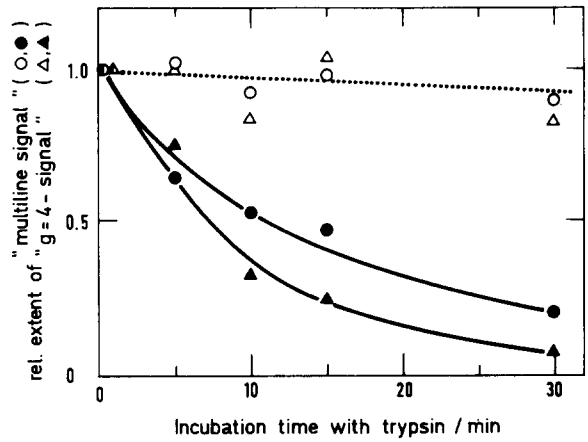


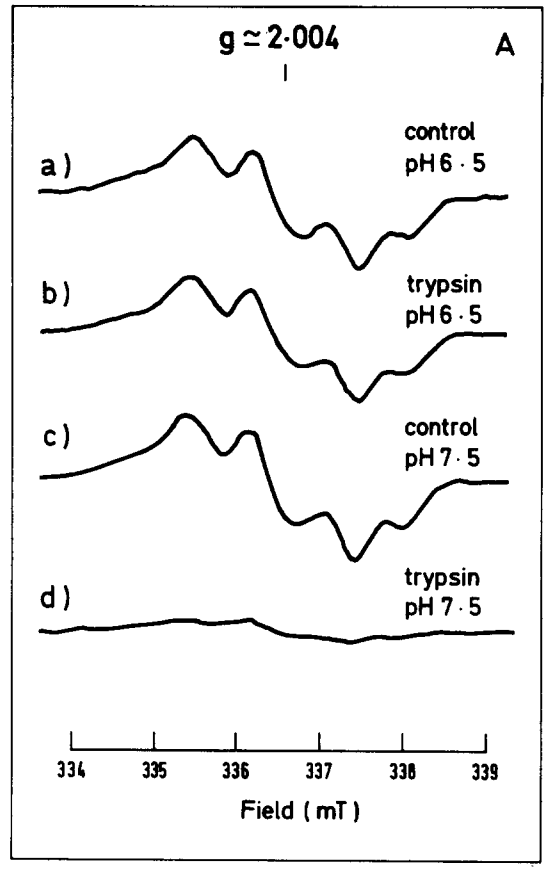
Fig. 2. A time-course of trypsin treatment on the multiline signal and the $g = 4.1$ signals. EPR signals were recorded as described in the legend to Fig. 1. These data are from an experiment in which the effect of trypsin was less rapid than that shown in Fig. 1. Samples were taken from the reaction mixture at various times during the course of trypsin treatment and were frozen in EPR tubes in the dark. The triangles represent the $g = 4.1$ signal and the circles represent the multiline signal. Open symbols were at pH 6.0, while solid symbols were at pH 7.5.

tion that DCMU addition resulted in loss of the $g = 4.1$, but not the multiline signal in samples illuminated at 200 K [12]. It was suggested that two turnovers were required to form the $g = 4.1$ signal in the presence of the multiline signal at 200 K [12]. Trypsin digestion of thylakoids is known to result in an inhibition of electron transfer from Q_A^- to Q_B [23]. Similar results were obtained for PS II-particles (Weiss, W. and Renger, G., unpublished results). Thus trypsin treatment at pH 6.0 should have the same effect as DCMU, i.e., the loss of the $g = 4.1$ signal due to the blockage of electron transport out of Q_A^-. Fig. 1 shows that, contrary to this prediction, the $g = 4.1$ signal is almost unaffected by trypsin at pH 6.0. The results do not support the proposal that the $g = 4.1$ signal arises from a component functioning between the S states and the reaction center. Since both the $g = 4.1$ signal and the multiline signal are present simultaneously in the same sample when a single turnover has taken place it must be concluded that the component giving rise to the $g = 4.1$ signal is either a donor on a side path which is oxidized at the expense of S_2 or arises from S_2 itself. Since this work was performed, in other EPR studies it was concluded that the $g = 4.1$ component and the multiline signal both arise from S_2 [24,25].

Signal II_{slow}

Signal II_{slow} probably arises from a semiquinone [26] which is the oxidized form of a component which is able to donate electrons to the PS II reaction center [27,28]. The donation reaction is slow (a few seconds) and is not thought to be on the reaction path between the S states and the reaction center [27,28]. This component is usually largely in the semiquinone form (i.e., oxidized) even after long dark adaptation. Since it is proposed to have an E_m of 750 mV [29] it is clearly out of equilibrium with the ambient redox potential. Trypsin treatment at pH 6.0 has virtually no effect on the signal, while at pH 7.5 trypsin treatment results in the loss of the signal (Fig. 3).

The effect of trypsin in this case is probably to expose the unstable free radical to the ambient potential with which it equilibrates. Little or no signal with the line-shape characteristic of Signal II could be generated in pH 7.5 trypsin-treated



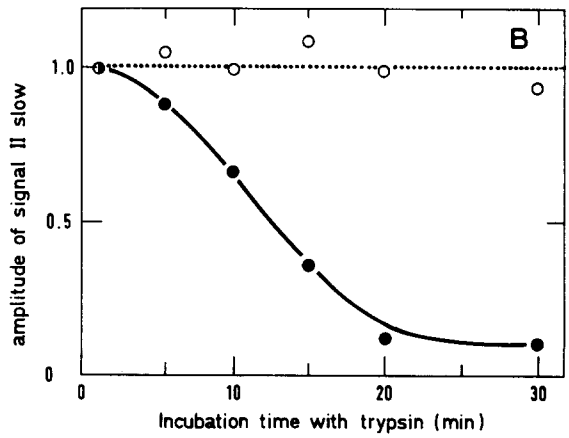


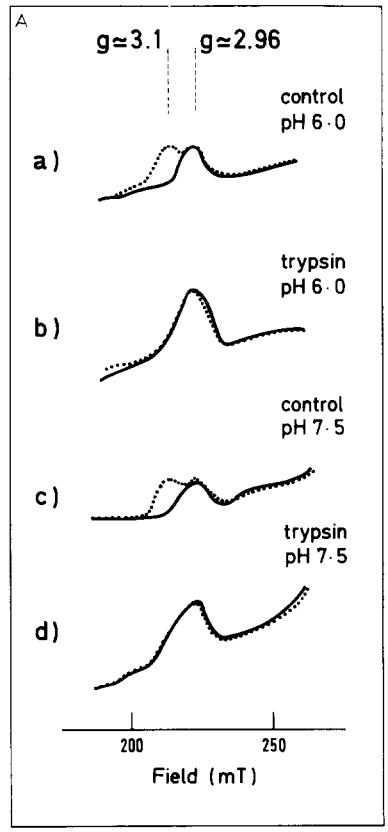
Fig. 3. The effect of trypsin on Signal II slow at pH 6.0 and pH 7.5. EPR conditions were as follows: temperature, 15 K, microwave power, 70 dB down from 200 mW; modulation amplitude, 2 gauss. (A) This shows the EPR spectra before and after trypsin treatment for 20 min at both pH values. In (B) a time-course experiment is represented, which is recorded in the same experiment shown in Fig. 2 and is directly comparable.

samples by illumination at 77 K, 200 K nor by freezing under illumination (not shown). However, we cannot rule out that the component responsible for Signal II is still photoactive after trypsin treatment. There are two reasons for this uncertainty: firstly, a modified signal might arise from this component after trypsin treatment which would not be recognized as Signal II; secondly, trypsin blocks electron transfer after Q_A^-, thus only a single electron will be extracted from the donor side and this electron does not necessarily come from the Signal II_{slow} component.

Cytochrome b-559

Cytochrome *b*-559 acts as a donor at low temperature but has an unknown function under physiological conditions [30]. The cytochrome is thought to exist in two redox forms, a high-potential and a low-potential form. These two forms give rise to EPR signal with slightly different *g*-values [31]. In the preparations of PS II particles used in this study the amplitude of the oxidized cytochrome signal (attributable to the low potential form, at $g = 2.94$) was about equal to that photoinduable at 77 K (the high-potential form at $g = 3.08$) (see Fig. 4a). Trypsin treatment at both pH values resulted in an increase in the amplitude of the oxidized low-potential form in the dark. This increase corresponded to a loss of the high potential form that can be photooxidized at low temperature (Fig. 4a). Time-course experiments showed that at low pH the conversion to the low-potential form was slightly slower than at high pH (Fig. 4b). But clearly cytochrome *b*-559 does not show the low pH immunity to trypsin attack enjoyed by the other donor side components studied here.

This could indicate that the effect of trypsin on Cyt *b*-559 is the result of an attack on the acceptor side of the membrane. In fact, it has already been shown that Cyt *b*-559 was more susceptible to trypsin-induced high-potential to low-potential transformation in right-side-out than in inside-out-thylakoids [32]. Alternatively, the effect on the cytochrome could be due to donor side proteolysis if the cytochrome was exposed to attack even at low pH. If this was the case it would indicate that the cytochrome protein is not surrounded by the manganese or the extrinsic 33 kDa polypeptide



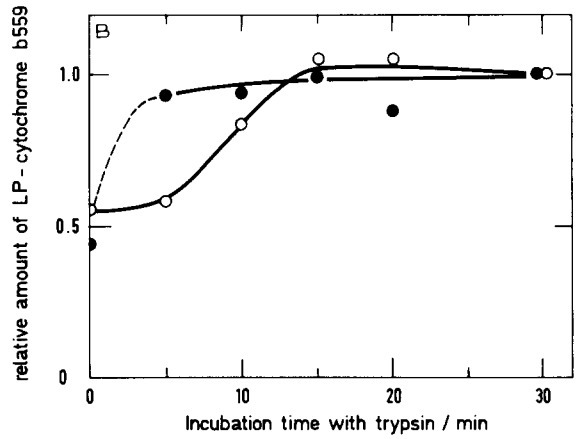


Fig. 4. The effect of trypsin on Cyt *b*-559 at pH 6.0 and pH 7.5. EPR conditions were as follows: temperature, 15 K; microwave power, 15 dB down from 200 mW; modulation amplitude, 20 gauss. (A) Solid lines show spectra recorded in the dark. Broken lines show spectra recorded after 20 min of illumination at 77 K. (B) A time-course experiment is represented which is recorded in the same experiment as shown in Fig. 2 and 3.

which are involved in O_2 evolution.

In a recent study a structural model for cytochrome *b*-559 was proposed in which the heme is liganded to histidines on different membrane spanning polypeptides [37]. In this model the *g*-value and redox properties of the high-potential form were proposed to arise from a situation in which the imidazole rings of the histidines were perpendicular to each other. Upon various treatments the change to the low potential form with its associated *g*-values was attributed to a relaxation of this structure to one in which the imidazole rings are parallel. The trypsin-induced high-potential to low-potential transition could be due to rapid digestion of exposed regions of the cytochrome polypeptides leading to such a relaxation of the structure within the membrane.

Since O_2 evolution is unaffected by the trypsination procedure used in this study at pH 6.0 [36], yet Cyt *b*-559 is converted to the oxidized low-potential form under these conditions, this represents further evidence (see also Refs. 32–34) that the high-potential form the cytochrome does not have an indispensable role in O_2 evolution in contrast to earlier suggestions [35].

After submission of this manuscript Cole et al. [38] reported a reversible decrease in the amplitudes of the multiline signal and the $g = 4.1$ signal when the pH was raised. The lack of such an effect in this study probably reflects the different experimental conditions used. In particular Cole et al. [38] used lower chlorophyll concentrations (1–2 mg Chl/ml), incubated for 1 h in ambient light under non-stacking conditions, whereas we have used high chlorophyll concentrations of stacked membranes incubated in darkness. The decrease of the EPR signals observed by Cole et al. [39] was discussed in terms of a replacement of Cl^- by OH^- assuming a specific role Cl^- in the function of the evolving enzyme (for a review see Ref. 39). In this respect, the loss of the multiline, the $g = 4$ signals and Signal II_{slow} by tryptic digestion at pH 7.5 could in part be due to a Cl^--depletion lesion due to digestion of a polypeptide which acts as a 'concentrator' of Cl^- (see Ref. 39).

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