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Photosystem II disorganization and manganese release after photoinhibition of isolated spinach thylakoid membranes

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The activity, the protein content and the manganese properties of photosystem II have been compared after photoinhibition of isolated thylakoid membranes. The results show a concomitant disappearance of the oxygen evolving activity and the ability to form the S_2-state multiline EPR signal. The D_1-protein is degraded in a subsequent event which closely correlates to release of manganese from the thylakoid membranes.

D_1-protein; EPR; Manganese; Photoinhibition, Photosystem II; Reaction center

1. INTRODUCTION

Photosystem (PS) II of higher plants is a multiprotein complex carrying a series of interconnected redox components [1,2]. Some proteins are inferred to have a direct catalytic function while others appear to have regulatory and/or structural roles. The isolation of a D_1/D_2/cytochrome *b*-559 protein complex competent in primary photochemistry of photosystem II [3] strongly supports the concept that the D_1- and D_2-proteins comprise the reaction center [4,5]. By analogy with the L and M reaction center polypeptides of photosynthetic purple bacteria it is thought that the D_1- and D_2-proteins are arranged as a heterodimer carrying all the redox components required for the photochemistry of PS II. In addition, the D_1- and D_2-proteins contain electron carriers at the oxidizing side of PS II. Iodination experiments [6] and site-specific mutagenesis [7,8] have identified the component D responsible for signal II_{slow} [2] as a tyrosine radical located in the D_2-protein. Circumstantial evidence suggests that the D_1- and/or D_2-proteins provide binding sites for the manganese ions required for water oxidation [9-11].

When the photosynthetic apparatus is exposed to strong light, PS II activity is inhibited [12]. The primary event of this photoinhibition is controversial [13], but it is known to induce degradation and resynthesis of the D_1-protein [14,15]. The consequence of this turnover for the protein organization and ligand binding of PS II is hard to envisage considering the central role of the D_1-protein.

In this study the combination of protein biochemistry and EPR spectroscopy has been used to correlate the inhibition of PS II activity with changes in protein composition and manganese content following photoinhibition of isolated thylakoid membranes.

2. MATERIALS AND METHODS

Thylakoid membranes were isolated from spinach leaves essentially as in [16] and suspended to a concentration of 150 μg chlorophyll/ml in 10 mM sodium phosphate, pH 7.4/5 mM NaCl/5 mM $MgCl_2$/100 mM sucrose. The thylakoid membranes were exposed to white light (7000 $\mu E \cdot m^{-2} \cdot s^{-1}$) under

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aerobic conditions at 20°C for the indicated periods of time. After illumination the thylakoid membranes were collected by centrifugation and resuspended to 6 mg chlorophyll/ml. Samples intended for low temperature EPR measurements were transferred to calibrated EPR tubes under dim light. Before being frozen the samples were incubated in the dark for 15 min at 20°C. Illumination of samples at 198 K was done in an unsilvered dewar by a 250 W lamp for 4 min, during which time the sample was immersed in a solid CO_2/ethanol bath.

SDS-polyacrylamide gel electrophoresis was carried out as previously described [17]. Western blotting, using monospecific antibodies against the D_1-, D_2- and 23 kDa proteins, was performed essentially according to [18] using ^{125}I-labelled protein A for detection. For quantification the autoradiograms were scanned by a laser densitometer.

Total manganese was determined by room temperature EPR spectroscopy. To release all manganese the samples were acidified with an equal volume of 2 M H_2SO_4. Calibration was done by addition of known amounts of $MnCl_2$.

Photosystem II mediated oxygen evolution was measured polarographically using phenyl-*p*-benzoquinone as the electron acceptor. The assay medium (1 ml) was composed of 30 mM sodium phosphate, pH 6.5/3 mM NaCl/60 mM sucrose/0.4 mM phenyl-*p*-benzoquinone and 20 μg chlorophyll.

Low temperature EPR spectra were recorded at X-band with a Bruker ER 200D-SRC spectrometer connected to an Aspect 2000 computer. The spectrometer was equipped with an Oxford Instruments cryostat and temperature controller. Normalization of the spectra for tube calibration factors and deviations in sample concentration were done with the computer. The S_2-state multiline EPR signal was measured at 10 K with a microwave power of 20 mW. The added amplitudes of three low field lines (indicated with arrows in fig.3) were used to compare the signal size.

3. RESULTS

Fig.1 shows the impairment of steady-state PS II activity after photoinhibitory illumination of isolated thylakoid membranes. The inhibition after 90 min of illumination was 95% and the inhibition half-time was approx. 20 min. This inhibition pattern was compared to changes in several PS II parameters. The disappearance of the D_1-protein was followed by quantitative immunoblotting (fig.2). The results show that the decrease in the amount of the D_1-protein was considerably slower than the inhibition of the PS II activity. There was only a marginal disappearance of the D_1-protein during the initial 15 min illumination although 40% of the activity was lost. After 90 min of illumination there was still approx. 50% of the protein present while the oxygen evolution was nearly completely inhibited. Immunoblotting of the photoinhibited thylakoids also revealed disappearance of the D_2-protein (fig.1) in agreement with recent

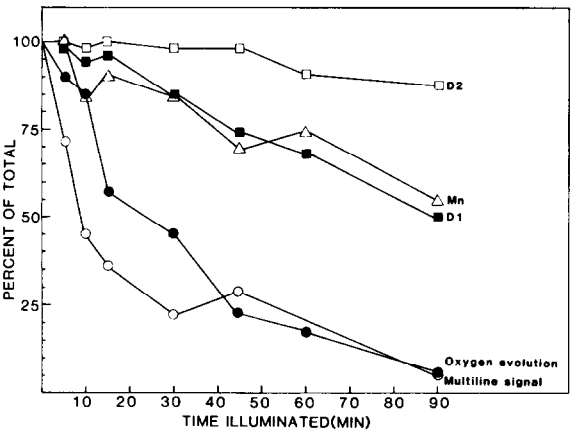

Fig.1. The effect of illumination at 20°C of isolated thylakoid membranes on oxygen evolution and various other components in PS II. (■) D_1-protein, (□) D_2-protein, (●) oxygen evolution, (○) formation of the S_2-state multiline EPR signal, (△) total content of manganese. In a control which was kept for 90 min in the dark at 20°C, the levels of the D_1-protein, the D_2-protein, oxygen evolution, multiline signal and total manganese were 96, 99, 98, 90 and 90%, respectively.

observations (Trebst, A., personal communication; [19]). However, the disappearance of the D_2-protein was slower than that of the D_1-protein.

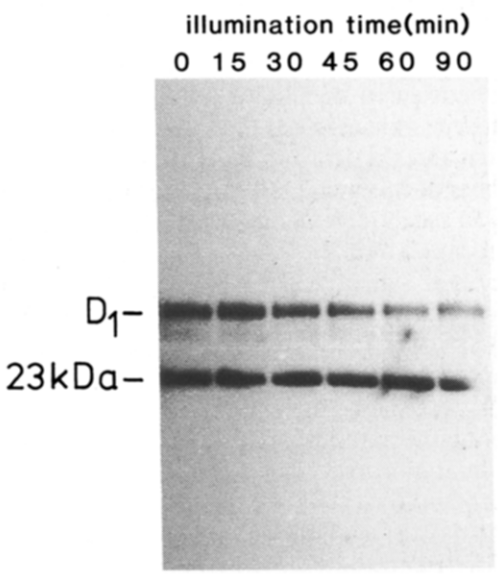

Fig.2. The effect of illumination at 20°C of isolated thylakoid membranes on the levels of the D_1-protein and the 23 kDa extrinsic protein as revealed by immunoblotting, using a mixture of antibodies against the two proteins.

Virtually no loss of the D_2-protein could be detected during the first 45 min of illumination and after 90 min as much as 85% of the protein remained (fig.1). Thylakoids kept in the dark at 20°C showed virtually unchanged levels of both the D_1- and D_2-proteins. The amount of the extrinsic 23 kDa PS II protein was not affected by the illumination (fig.2) and could therefore be used as an internal standard in the immunoblotting experiments. Moreover, immunoblotting revealed unchanged levels of the 9 kDa subunit of cytochrome *b*-559 and the 22 kDa integral protein described by Ljungberg et al. [17]. Thus, photodamage of PS II is located to the reaction center proteins.

No degradation products of the D_1-protein could be detected in the immunoblotting analysis (fig.2). Nevertheless, when [^{35}S]methionine labelled thylakoids were used in an experiment similar to that presented in fig.1, increased levels of radioactivity were found in the supernatants after pelleting the photoinhibited thylakoid samples (not shown). It therefore appears that the efficient degradation system of the D_1-protein [20,21] is operational in isolated thylakoid membranes and therefore likely to be membrane bound. In contrast, there was virtually no D_1-protein degradation in Triton-derived PS II particles (BBY) although the rate of inhibition of oxygen evolution was approximately the same as for the isolated thylakoids (unpublished).

The possible connection between the manganese cluster and the D_1- and D_2-proteins prompted an analysis of this metal after photoinhibition. Under normal conditions, illumination at 198 K results in the formation of the S_2-state multiline EPR signal [22]. This signal originates from the manganese cluster of the oxygen evolving system and can be used as a spectroscopic probe for its functional intactness. Low-temperature EPR spectroscopy (fig.3) revealed impairment of the ability to generate the multiline signal after photoinhibition. The inhibition preceded the disappearance of the D_1-protein (fig.1) but correlated with the inhibition of the oxygen evolution. Only one electron can be transferred to the Q_A-Q_B acceptor complex by illumination at 198 K and the electron is normally donated by the water splitting system. When this is inhibited the electron is taken from cytochrome *b*-559 or a chlorophyll molecule [23]. However, no

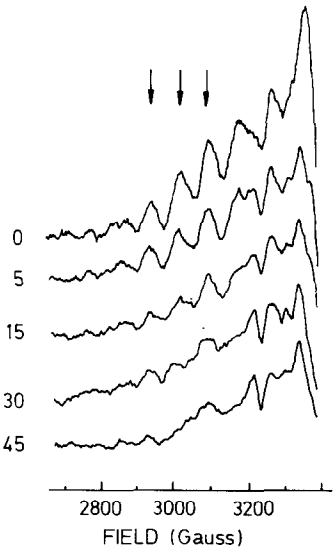

Fig.3. EPR spectra showing the low field part of the S_2-state multiline EPR signal. The signal was induced by continuous illumination at 198 K of photoinhibited thylakoid membranes illuminated for various times as indicated. The added amplitudes of the peaks marked with arrows were used to compare the signal size in different samples.

oxidation of either cytochrome *b*-559 or a chlorophyll could be observed by EPR spectroscopy when the formation of the multiline signal was impaired by photoinhibition (not shown).

The total amount of manganese in the thylakoid membranes decreased during photoinhibition (fig.1). Strikingly, the release of manganese from

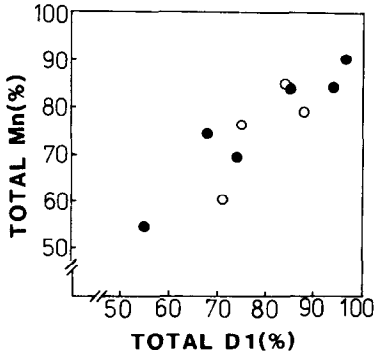

Fig.4. The fraction of manganese remaining the photoinhibited thylakoid membranes plotted as a function of the fraction of remaining D_1-protein. Data from two sets of experiments are shown (closed symbols are from the experiment in fig.1).

the membranes correlated closely to the degradation of the D_1-protein (figs 1 and 4). The release of the manganese was not accelerated by the subsequent disappearance of the D_2-protein (fig.1). The total loss of manganese in the experiment shown in fig.1 represented 2 manganese ions/reaction center of PS II (assuming 500 chlorophyll molecules/PS II) corresponding to 4 manganese ions released per D_1-protein degraded.

4. DISCUSSION

The D_1-protein degradation was considerably slower than the inhibition of the oxygen evolution. This clearly shows that photoinhibition primarily destroys the photochemical reactions and that protein degradation is a subsequent event in agreement with [13,20].

The correlation between the release of manganese from the thylakoid membrane and the degradation of the D_1-protein is intriguing (fig.4). The most straightforward interpretation is that manganese ions are bound to the D_1-protein and lost from the active site on the lumenal side of the thylakoid membrane due to protein degradation. The ions subsequently leak through the membrane. According to the current ideas, the oxygen evolving system contains four manganese ions two of which are more easily extracted than the others [22]. The described correlation (fig.4) is easily understood if all four manganese ions are lost simultaneously when the D_1-protein is destroyed. This then suggests that the four manganese ions all are directly or indirectly associated with the D_1-protein. In the latter case they would presumably be lost from their binding site (on another protein) due to a general disassembly of PS II when the D_1-protein is removed from the reaction center. The present data do not allow discrimination between intra- or interprotein ligation of manganese ions. However, manganese binding at the interface between two proteins has been proposed from spectroscopic analogies with the Fe-protein in nitrogenase [24] and from extensions of the D_1/D_2-heterodimer concept [9–11]. If manganese ions are shared between the D_1-protein and another protein, a likely candidate is either the D_2-protein, that comprises the reaction center together with the D_1-protein, or another subunit in oxygen evolving core particles [1]. Earlier suggestions often centered on the extrinsic 33 kDa protein as a manganese-protein [11,22,25], but several lines of evidence argue against this hypothesis [1,26,27].

The formation of the multiline signal by illumination at 198 K requires only one stable charge separation and it is likely that all centers capable thereof turn over in the strong light applied. On the other hand, steady-state oxygen evolution measurements require hundreds of turnovers. Therefore the correlation between the impairment of the multiline signal formation and the inhibition of oxygen evolution (fig.1) strongly suggests that the inhibition resulted from an inability to perform stable charge separations rather than slowing down one or several steps in the electron transfer between water and the electron acceptor. This conclusion is further strengthened by the observation that neither cytochrome *b*-559 nor chlorophyll donated electrons when the oxygen evolving system was inhibited. A more complete characterization of the chemical events following photoinhibition using EPR spectroscopy is in progress.

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