Volume 280, number 1, 87–90 FEBS 09466 March 1991

ADONIS 0014579391001768

Fast oxygen-independent degradation of the D1 reaction center protein in photosystem II

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Received 12 December 1990; revised version received 17 January 1991

The D1 reaction center protein in photosystem II is rapidly degraded during illumination of chloride-depleted or Tris-washed thylakoids. The degradation is independent of oxygen and occurs under anaerobic conditions provided that electrons can flow through the acceptor-side of photosystem II. This shows that oxygen-derived reactive species are not necessarily involved in the light-dependent damage of the D1 protein. Instead the illumination of chloride-depleted or Tris-washed thylakoids induces long-lived, strongly oxidizing radicals on the donor-side of photosystem II which are suggested to be the damaging species for the D1 protein.

Photosystem II; D1 protein; Photoinhibition; Protein degradation

1. INTRODUCTION

Photosystem II catalyzes the light-driven reduction of plastoquinone with electrons derived from water (for reviews see [1,2]. The reaction center in PSII is a heterodimer of two membrane-spanning proteins, D1 and D2 [3–5], which is surrounded by more than 20 other structural, light-harvesting and regulatory subunits [1]. The D1/D2 heterodimer binds all the redox components involved in the primary photochemistry and probably also the Mn-cluster in the oxygen evolving complex [1]. It is therefore intriguing that the D1 protein turns over in the light faster than any other thylakoid protein [6–8]. The turnover cycle [9] of the D1 protein starts with light-dependent triggering of the D1 protein for degradation. Thereafter, the damaged protein is degraded by at least one specific membrane-bound protease [10–13]. New copies of the D1 protein are subsequently incorporated together with the other PSII subunits into functional PS II complexes [9].

The degradation of the D1 protein has been studied in thylakoids [10,14], PSII-enriched membranes [15,16], inside-out vesicles [13] and core complexes of PSII [11]. The turnover of the D1 protein has been coupled to the light-dependent reactions that lead to photoinhibition of the electron transport through PSII [6] but the event that trigger the D1 protein for degradation is not understood. In preparations capable of oxygen-evolution, photoinhibition is due to over-reduction of the acceptor side in PSII ([6,15] and references cited therein). Under these conditions it is generally thought that oxygen-derived, reactive species are responsible for the damage to the D1 protein [16–20]. This is supported by experiment using synthetic oxygen-radical producing systems [20]; Aro, Hundal, Carlberg and Andersson, unpublished results) and the lack of degradation of the D1 protein in the absence of oxygen [16,19].

When compared to oxygen-evolving preparations, the D1 protein is much more light-sensitive when the water-oxidizing complex is inhibited by hydroxylamine treatment (which removes the Mn-cluster) [21] or by Cl^--depletion (which reversibly inhibits the oxygen-evolution) [14]. This increased light-sensitivity of the D1 protein is most probably [14,22,23] due to the accumulation of strongly oxidizing radicals on the donor-side of PSII (Tyr_Z^+ and/or P_{680}^+) which occur when the electron donation to P_{680}^+ is slowed down but sofar the reactive species has not been conclusively identified.

It is probable that the endogenic oxidizing radicals in PSII damage the D1 protein via a reaction mechanism that differs from the oxygen-dependent damage to the D1 protein which occurs as a consequence of reactions at the acceptor-side of PSII. Therefore, we have investigated the oxygen dependence of the fast degradation of the D1 protein in thylakoids with an inhibited donor side. It is shown that the D1 protein is very light-sensitive and can be rapidly degraded under both aerobic and anaerobic conditions. Mechanistic aspects of different reactions leading to damage of the D1 protein will be discussed.

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPC, 2,2′-diphenylcarbonic dihydrazide; P_{680}, primary electron donor chlorophyll(s); PSII, photosystem II; Q_A, first quinone acceptor in PS II; Q_B, second quinone acceptor in PSII; Tris, tris(hydroxymethyl)aminomethane; Tyr_Z, tyrosine-161 on the D1 protein – electron carrier between the Mn-cluster and P_{680}

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2. MATERIALS AND METHODS

Thylakoids inhibited on the donor side by Cl^--depletion and Tris-washing were prepared according to [14]. The degree of Cl^--depletion, assayed as oxygen evolution in the absence or presence of 20 mM NaCl, was 60–70%. Electron transfer from water to DCIP was measured as in [14] in the presence of 40 mM NaCl. Electron transfer from DPC to DCIP was measured using the same buffer in the presence of 1 mM DPC.

Photoinhibitory illumination was performed as in [14], aerobically or anaerobically in a medium composed of 40 mM sodium-phosphate buffer at pH 7.4, 100 mM sucrose, 5 mM glucose and 5 mM $MgSO_4$. In the anaerobic experiments 20 mM $NaHCO_3$ was added to the photoinhibition buffer to eliminate inhibitions at the acceptor-side of PSII induced by the anaerobic conditions [24]. The activity measurements were performed immediately while the samples intended for the D1 protein analysis were frozen on dry-ice. Thereafter all sample-handling was performed in darkness.

Oxygen-free conditions were obtained by a combination of argon-flushing and enzymatic removal of oxygen. The buffer solution was bubbled with argon for 5 min perior to the addition of glucose oxidase (0.4 mg/ml; Sigma) and catalase (≈ 4000 units/ml; Sigma). The argon bubbling in the suspension was stopped, but a gentle stream of argon was continuously blown over the surface of the solution. Thereafter the thylakoids and bicarbonate (when used) were added from concentrated stock solutions (total addition approximately 10% (v/v)). Within one minute after the addition of the coupling enzymes less than 2 μM (our detection limit) oxygen remained in the reaction medium. Prior to the illumination the solution was stirred gently for 5 min to allow equilibration with the argon atmosphere above the reaction vessel. The reason for using an enzymatic oxygen scavenger system instead of flushing or pumping methods was that this system rapidly reduces any oxygen formed in the photosynthetic reactions during the illumination.

SDS-polyacrylamide gel electrophoresis and quantitative Western blot analysis of the thylakoid proteins were performed as described earlier [10] using specific antibodies against the D1 and 22 kDa proteins. In the analysis of the degradation of the D1 protein the 22 kDa protein in PSII was used as an internal standard, as this protein does not change markedly during photoinhibition [10].

3. RESULTS

3.1. *Photoinactivation of electron transfer reactions in Cl^--depleted thylakoids under aerobic and anaerobic conditions*

Fig. 1A shows the results of photoinactivation (performed in the absence of Cl^-) of the electron transfer from water to DCIP in Cl^--depleted thylakoids. Under aerobic conditions (Fig. 1A, closed triangles) the oxidation of water is rapidly inhibited by the illumination in approximately 60% of the centers ($t_{1/2} \approx 2.5$–3 min for the inhibition) while the remaining part of the centers (presumably centers that had not lost Cl^- during the depletion procedure [14]) were inhibited with slower kinetics. Under anaerobic conditions (Fig. 1A, open circles) the Cl^--depleted thylakoids were also rapidly inhibited ($t_{1/2} \approx 2.5$ min for the inhibition). The kinetics are similar to the aerobic case but in the anaerobic experiment the fast inhibition involves all centers. This difference from the aerobic experiment is probably due to the presence of bicarbonate since also under aerobic conditions the rapid photoinactivation involves all centers in the presence of bicarbonate (not shown).

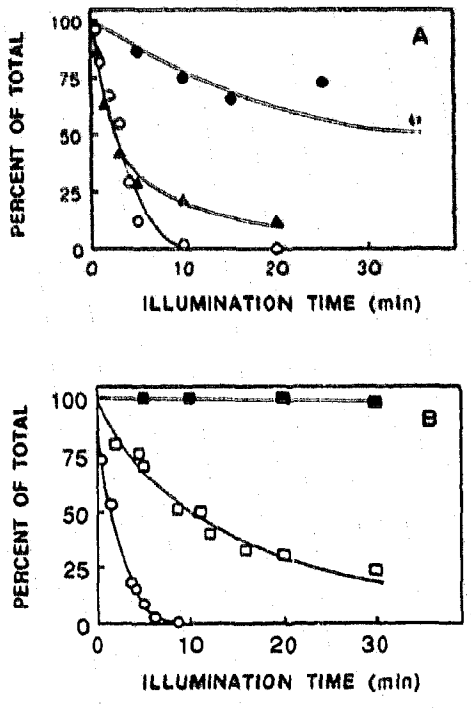

Fig. 1. Effect of illumination (2900 $\mu E \cdot m^{-2} \cdot s^{-1}$) of Cl^--depleted thylakoids (600 μg chlorophyll · ml^{-1}). (A) Light-dependent inhibition of the electron transfer from water to DCIP under aerobic conditions (closed triangles) and anaerobic conditions (open circles). The filled circles represent an anaerobic sample kept in the dark. (B) Light-dependent inhibition of the electron transfer from DPC to DCIP (open squares) and water to DCIP (open circles) under anaerobic conditions. The filled squares represent the electron transfer between DPC and DCIP in an anaerobic sample kept in the dark.

The electron-transfer between DPC and DCIP (from Tyr_Z to the Q_B site) is inhibited with 7–8 times slower kinetics than the water oxidation under anaerobic conditions (Fig. 1B). This relative difference in light sensitivity for the two electron transfer reactions is constant under aerobic or anaerobic conditions (Fig. 1A and B) and at different light intensities (compare Fig. 1B with the results in [14] that were obtained at a lower light-intensity). In a dark control the water oxidation is partially lost (Fig. 1A, closed circles) during the time course for the experiment while the electron transfer from DPC to DCIP is stable (Fig. 1B). The stability of the latter reaction implies that also the D1 protein is stable in the dark.

3.2. *Degradation of the D1 protein in the absence of oxygen*

The D1 protein in Cl^--depleted thylakoids is very sensitive to illumination under aerobic conditions [14] and in this study we have investigated the effect of oxygen on this reaction. When Cl^--depleted thylakoids are illuminated under anaerobic conditions, 85% of the D1 protein is degraded in 60 min (Fig. 2A, lanes 1–4). The anaerobic degradation of the D1 protein occurs

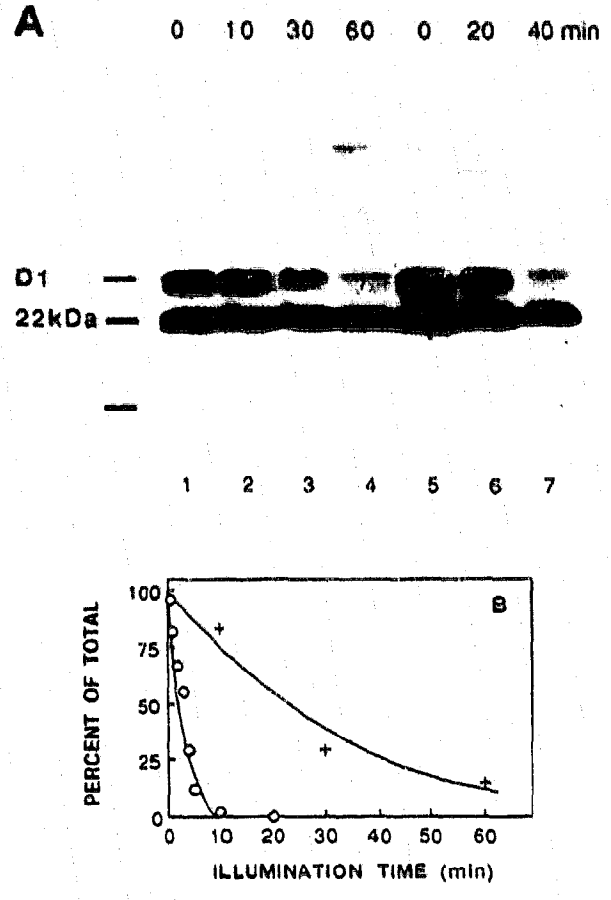

Fig. 2. Light-dependent degradation of the D1 protein under anaerobic conditions. (A) Immunoblot of PSII proteins from an experiment in which Cl^--depleted thylakoids (lanes 1–4) or Tris-washed thylakoids (lanes 5–7) were illuminated for different times under anaerobic conditions. Immunoblotting were performed with antibodies against the D1 protein and the 22 kDa protein. The bar indicates the location for a peptide that crossreacts with the D1 antibody. The Cl^--depleted membranes were illuminated as in Fig. 1. The Tris-washed membranes were illuminated with 1900 $\mu E \cdot m^{-2} \cdot s^{-1}$ at a chlorophyll concentration of 100 $\mu g \cdot ml^{-1}$. (B) Kinetics for the anaerobic degradation of the D1 protein (crosses) and inhibition of the electron transfer from water to DCIP (open circles) in Cl^--depleted thylakoids. The D1 protein data are from the immunoblot in Fig. 2A (lanes 1–4) and the electron-transport data are from Fig. 1A (open circles).

with 7–8 times slower kinetics than the inhibition of the oxygen evolution (Fig. 2B). Thus, the degradation of the D1 protein and the inhibition of the electron transfer between DPC and DCIP (Figs 1B and 2B) are quite closely correlated under anaerobic conditions, similar to what was found under aerobic conditions [14]. We also observed fast degradation of the D1 protein when Tris-washed thylakoids were illuminated under anaerobic conditions (Fig. 2A, lanes 5–7).

In the experiment with Cl^--depleted thylakoids (Fig. 2A, lanes 1–4) a peptide with an apparent molecular mass around 12–15 kDa cross-reacts with the D1-antibody (indicated with a bar in Fig. 2). This peptide is formed during the illumination since it only present in the samples that were illuminated for longer times and its appearance is correlated with the disappearance of the D1 protein. In the 60 min sample the peptide corresponds to approximately 5% of the total D1 protein immunoresponse present from the start. The fragment observed here (Fig. 2A) is of similar size to a fragment described earlier in PSII core particles [11] and in vivo [25] but whether it represents an identical peptide remains to be established.

4. DISCUSSION

The studies of the degradation of the D1 protein have been concentrated on high illumination of photosynthetic systems with a functional oxygen-evolving complex. In this case it is thought that reactive oxygen-derived species are important in the reactions that trigger the D1 protein for degradation [16,19]. This is supported by experiments using hydroxyl radical-generating systems and radical scavengers ([20,26]; Aro, Hundal, Carlberg and Andersson, unpublished results).

The D1 protein is extremely sensitive to illumination when the oxygen-evolving complex is unable to rapidly reduce the oxidizing radicals (P_{680}^+ and/or Tyr_Z^+) on the donor side of PSII [14,21]. Here we demonstrate that the fast degradation of the D1 protein in such systems is independent of oxygen. This result is important since it clearly shows that there exist two different light-dependent reaction mechanisms that damage the D1 protein and trigger it for protein degradation.

This conclusion raises interesting questions. By which mechanism is the D1 protein triggered for degradation in each case? Are both mechanisms equally applicable to the turnover of the D1 protein in vivo?

In oxygen-evolving preparations, photoinhibition is probably due to the double reduction of Q_A [15]. In this case there exists at least two plausible mechanisms for the formation of reactive, oxygen-derived species that might be involved in the damage to the D1 protein. Oxygen radicals have been suggested to be formed in reactions between acceptor side components in PSII and oxygen. In secondary reactions these radicals would then damage the D1 protein [6,18]. An alternative mechanism involves singlet-oxygen which is very oxidizing [27]. In photosynthetic systems, a known origin for singlet oxygen is the reaction between $^3P_{680}$ and molecular oxygen. $^3P_{680}$ may be formed in the back-reaction between pheophytin$^-$ and P_{680}^+ when Q_A is non-functional [26] and it is known [29–31] that light-dependent singlet oxygen formation rapidly inhibits the photochemistry in the D1/D2/cytb_{559} reaction center preparation which lacks Q_A [5].

A different hypothesis implies that the light-sensitivity of the D1 protein is a mere consequence of the oxidative chemistry involved in the oxidation of

water [14,22]. In this model, PSII may be damaged also at low light intensities since there is always a certain probability that P_{680}^+ or Tyr_Z^+ which are both very oxidizing, and are not reduced fast enough by electrons from water. In support of this hypothesis it has been found that the D1 protein is extremely light-sensitive when the oxygen-evolving complex is inhibited [14,21,23]. The reason is proposed to be [14,22] that the lifetime for P_{680}^+ and Tyr_Z^+ increases when the water-oxidizing complex is inhibited. This leads to the abnormal accumulation of these highly oxidizing radicals on the donor side of PSII, which then directly might oxidize the protein or closely situated redox-components. This hypothesis is strongly supported by our present results which show that oxygen-derived species are not involved in the damage to the D1 protein when the water-oxidizing complex is inhibited.

The question of which mechanisms apply to the D1 turnover in vivo is important. The turnover of the D1 protein in vivo also occurs at low light-intensities [7,8] where it seems unlikely that the acceptor-side of PSII should continuously become overreduced in a significant fraction of the centers. Instead it is possible that the protein turnover under these conditions is dominated by damage induced by the donor-side radicals. Some possible lesions in the oxygen-evolving complex that might slow down the reduction of P_{680}^+ and/or Tyr_Z^+ have been discussed earlier [14]. However, it is reasonable that strong illumination might induce over-reduction of the acceptor-side also in vivo and it is possible that under such circumstances oxygen-derived, reactive species might be formed which interact specifically with the D1 protein.

In oxygen-evolving thylakoids, anaerobic conditions result in very rapid photoinhibition of the electron transport [19,32,33] due to the formation of an unusually stable form of reduced Q_A [33]. This fast photoinhibition is eliminated by the presence of bicarbonate during the illumination due to reactivation of the acceptor-side by an as yet undefined mechanism [24]. In Cl^--depleted thylakoids we have observed a similar phenomenon (i.e. rapid build-up of reduced Q_A) under anaerobic conditions and to avoid acceptor-side limitations we performed the experiments presented here in the presence of bicarbonate. The effects of bicarbonate on the photoinactivation of Cl^--depleted thylakoids will be described elsewhere.

Acknowledgements: The skilful and engaged technical assistance by Mrs. A.-M. Tomka is gratefully acknowledged. We appreciate the useful discussions with Drs B. Andersson, H. Salter, I. Vass and I. Virgin. The D1 antibodies were a kind gift from Dr I. Ohad (Israel). This work was supported by the Swedish Natural Science Research Council and the Erna and Victor Hasselblad Foundation. S.S. was supported by a long-term grant for biotechnological basic research financed by the Knut and Alice Wallenberg Foundation.

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