Volume 287, number 1,2, 125–128 FEBS 09996 August 1991

ADONIS 001457939100707M

Light-induced D1 protein degradation is catalyzed by a serine-type protease

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Received 2 June 1991

Light-induced degradation of the D1 protein in isolated spinach photosystem II core preparations was studied after addition of various protease inhibitors. The degradation was selectively inhibited by several serine protease inhibitors in particular diisopropylfluorophosphate. The results demonstrate that the D1 protein is degraded by a serine-type of proteolytic activity that is an integral part of photosystem II.

D1 protein; Photoinhibition; Photosystem II; Protease inhibitor; Protein turnover; Serine protease

1. INTRODUCTION

The D1 protein turns over at a considerably higher rate than any other subunit of the photosystem II complex [1,2]. This high turnover and its relevance for the repair of light-induced inhibition of photosystem II electron transport are presently the subject of intense research. The molecular mechanism for the various steps in the turnover, including the D1 protein degradation itself, is largely unknown. The degradation can be divided into two principal steps [3]. The first step is triggering of the D1 protein for degradation as a direct consequence of light-induced impairment of photosystem II electron transport. This triggering is thought to occur through photosystem II-mediated formation of toxic oxygen species [4–8] or radicals formed directly in the reaction centre [9–11]. In the second step the triggered D1 protein is proteolytically cleaved to allow for replacement of the damaged protein by a newly-synthesized copy. The enzymatic nature of the degradation is supported by its temperature dependence [12,13] and by the fact that it per se does not require light [13].

Most studies on the degradation of the D1 protein have been performed in vivo [1,2]. However, a number of studies have shown that D1 protein degradation can also occur in vitro [3] as a consequence of photoinhibitory illumination of isolated thylakoids [12–14], photosystem II membranes [15,16], photosystem II core complexes [17] and even purified photosystem II reaction centres [18,19].

Taken together, these observations strongly suggest that the proteolytic activity responsible for degradation of the D1 protein is an integral part of photosystem II and confined to the reaction centre itself. In this study we have further analyzed the nature of this enzymatic activity using various classes of protease inhibitors. The results demonstrate that the D1 protein is cleaved by a serine-type of protease activity.

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Abbreviations: pA-PMSF, [4-amidino-phenyl]-methane-sulphonyl fluoride; chl, chlorophyll; DFP, diisopropylfluorophosphate; EDTA, ethylene diaminetetraacetate, disodium salt; MES, 2-morpholino-ethanesulphonic acid-1-hydrate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate.

2. MATERIALS AND METHODS

Thylakoid membranes were isolated from spinach leaves essentially as in [20]. Photosystem II core complexes were purified from a photosystem II membrane subfraction as described in [21] and suspended in 50 mM MES, pH 6.0, 10 mM NaCl, 0.4 M sucrose. In addition 2 mM ferricyanide was present. Protease inhibitors were obtained from Boehringer with the exception of diisopropylfluorophosphate which was obtained from DuPont. Concentrations of added inhibitors were essentially two times those the supplier recommended. Diisopropylfluorophosphate was used at the concentration of 0.1 μM. Subsequent photoinhibitory treatment was done by illuminating samples [100 μg chl·ml^{-1}) with white heat-filtered light [7000 μE·m^{-2}·s^{-1}) for 30 min at room temperature under aerobic conditions.

SDS-PAGE was carried out essentially as in [22]. Immunoblotting onto PVDF membranes was performed essentially as in [23] using a monospecific antisera against the D1 protein. For detection, ^{125}I-labelled protein-A was used and subsequent quantification was performed by scanning the autoradiograms with a laser densitometer.

3. RESULTS

In order to further characterize the enzymatic

mechanism for D1 protein degradation, the proteolytic activity was classified using various kinds of protease inhibitors. In initial experiments, protease inhibitors were added to isolated thylakoid membranes prior to photoinhibitory illumination. Such illumination, without addition of inhibitors, normally results in at least 30% degradation of the D1 protein [14]. Inhibitors pA-PMSF (serine proteases), bestatin (amino peptidases) and pepstatin (aspartate proteases), individually added to thylakoid membranes, caused virtually no inhibition of this degradation (not shown). Addition of a protease inhibitor cocktail (Boehringer) containing 10 different compounds (antipain, pA-PMSF, aprotinin, bestatin, chymostatin, E-64, EDTA, leupeptin, pepstatin and phosphoramidon), inhibited the reaction by less than 10%.

In the next set of experiments the inhibitor studies were performed using isolated photosystem II core complexes, in which D1 protein degradation can easily be obtained [17]. Only 20% of D1 protein remained after 30 min of photoinhibitory illumination (Fig. 1, lanes 1 and 7). Addition of the protease cocktail drastically reduced the observed degradation, with as much as 60% of the original level of D1 protein remaining. Addition of the cocktail therefore results in 50% inhibition of the degradation reaction (Table I). The discrepancy in inhibitor effect between thylakoids and isolated photosystem II core complexes can most likely be explained in terms of accessibility.

Table I

Classification of the proteolytic activity responsible for light-induced D1 protein degradation in photosystem II core complexes using protease inhibitors

Protease Inhibitor		
Compound	Class	Inhibition [%]
+ Cocktail*	All	50
+ EDTA	Metallo	0
+ E-64	Thiol	0
+ Phosphoramidon	Metallo	0
+ Bestatin	Amino	10
+ pA-PMSF	Serine	40
+ Antipain	Serine	50
+ Aprotinin	Serine	45
+ Diisopropylfluoro-phosphate	Serine	60

The effect of each of the indicated protease inhibitors on light-induced D1 protein degradation was quantified by immunoblotting. In control illuminated photosystem II core complexes without additions, 20% of the D1 protein remains after 30 min of photoinhibitory illumination. The effect of each inhibitor is expressed as the percentage of this degradation reaction that was inhibited.

* Cocktail contained 10 protease inhibitors as described in the text.

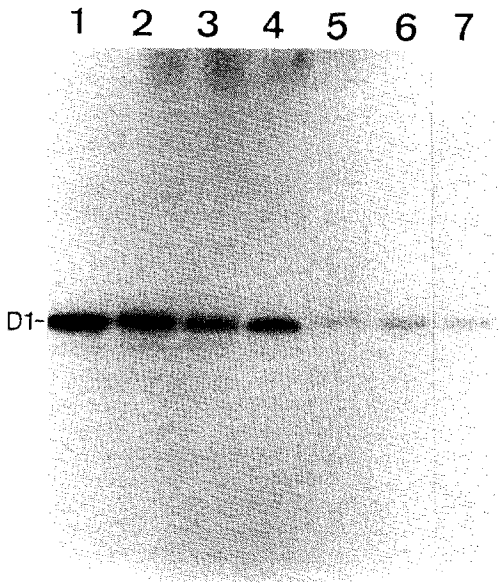

Fig. 1. Immunoblot showing the relative amounts of D1 protein in isolated PSII cores after illumination at 7000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 30 min in the presence or absence of the indicated protease inhibitors. Lane 1, dark control, no additions; lane 2, antipain, 100 $\mu g \cdot ml^{-1}$; lane 3, aprotinin, 0.6 μM; lane 4, pA-PMSF, 40 μM; lane 5, EDTA, 2.7 mM; lane 6, bestatin, 260 μM; lane 7, illuminated control, no additions. Following illumination, the proteins were separated on a 12–22.5% gradient SDS-PAGE system in the presence of 4 M urea and immunodecorated with anti-D1 protein sera. The position of the mature D1 protein (32 kDa) is indicated. The amount of material applied to the gel for quantitation is too low to detect the proteolytic fragments of the D1 protein [17].

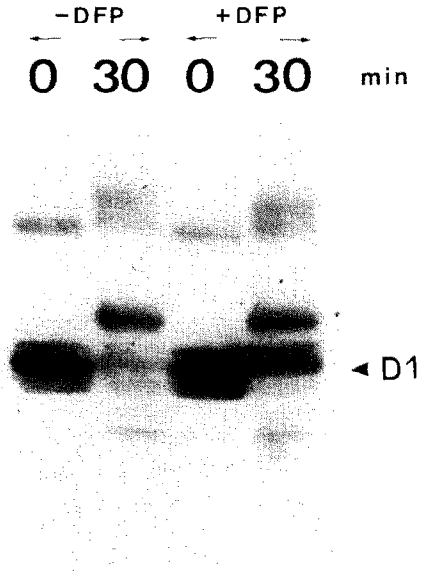

Fig. 2. Immunoblot showing the relative amounts of D1 protein in isolated PSII cores after illumination at 7000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 30 min in the presence or absence of the serine protease inhibitor diisopropylfluorophosphate (0.1 μM). The reaction mixture contained 200 mM NaCl, 20 mM Tris-HCl, pH 7.5. From left to right, the lanes represent 0 and 30 min illumination without inhibitor (−DFP) and 0 and 30 min illumination in the presence of inhibitor (+DFP). Following illumination, the proteins were separated on a 12–22.5% gradient SDS-PAGE system in the presence of 4 M urea and immunodecorated with anti-D1 protein sera. The position of the mature D1 protein (32 kDa) is indicated. The 23 kDa proteolytic fragment and the D1 fragment/D2 protein sub-heterodimer [17] are visualised.

This experiment, from a conceptual view, strongly corroborates evidence for the proteolytic nature of the D1 protein degradation process, and from an experimental point of view provides a guideline to the identification of the active class of protease inhibitor. Consequently, the experiments were continued by the addition to isolated photosystem II core complexes of individual protease inhibitors from the cocktail used above. As shown in Fig. 1, addition of EDTA (lane 5) and bestatin (lane 6) caused no or very little inhibition of D1 protein degradation during strong illumination (Table I). Moreover, no inhibitory effect was seen with the thiol protease inhibitor E-64 or the metalloprotease inhibitor phosphoramidon (Table I). In contrast, as shown in Fig. 1, antipain (lane 2), aprotinin (lane 3) and pA-PMSF (lane 4), all inhibitors of serine proteases, caused a significant reduction of D1 protein degradation. Quantification showed the degree of inhibition caused by these three inhibitors to be approximately 40–50% (Table I).

The results presented in Table I suggest that the mechanism for light-induced D1 protein degradation involves a serine protease. In order to conclusively establish this, we added diisopropylfluorophosphate to isolated photosystem II core complexes. This compound is unique and diagnostic for serine proteases, forming a covalent adduct with the reactive serine γ-O side chain of the protease active site [24]. The complexes were therefore incubated with 0.1 μM diisopropylfluorophosphate at 4°C, as described in [25], and then subjected to photoinhibitory illumination for 30 min at 20°C. As shown in the immunoblot of Fig. 2, the presence of diisoprofluorophosphate gave rise to significant reduction in the degree of D1 protein degradation. Quantification revealed as much as 60% inhibition (Table I) thereby identifying diisopropylfluorophosphate as the most efficient protease inhibitor tested. Inhibition of degradation remained after spinning the photosystem II core complexes out from the incubation medium. This indicates an inhibition mechanism that involves binding of diisopropylfluorophosphate to a photosystem II protein subunit.

4. DISCUSSION

The present experiments on light-induced D1 protein degradation using various classes of protease inhibitors are strong evidence for the involvement of a serine-type protease. In particular, inhibition by the taxonomic serine protease inhibitor diisopropylfluorophosphate via binding to the isolated photosystem II complex is conclusive. The involvement of a serine protease in the D1 protein degradative process is at variance with [26] where a role for an thiol peptidase was suggested.

This serine type of proteolytic activity must be located within the photosystem II complex [17]. Very recent results from our own group [18] and work presented in [19] suggest that the proteolytic activity is present even in reaction centre preparations containing only the D1 and D2 proteins, the two subunits of cytochrome *b*-559 and the *psb*I gene product [27]. The possibility that the D1 protein may be degraded by a co-purifying and contaminating serine protease is highly unlikely. First of all the degradation does not occur in the dark and has to be induced by photoinhibitory illumination. Moreover, the degradative pattern is very reproducible, and occurs to the same extent, per photosystem II complex, at different dilutions of these isolated complexes. Finally, further subfractionation of photosystem II core complexes through several chromatographic purification steps did not change the fragment pattern seen following photoinhibitory illumination. The fact that the proteolysis gives rise to fragments [17] similar to those seen in vivo [2] strongly supports the physiological significance of the present observations.

The involvement of a serine protease should have significant relevance for understanding the molecular mechanism of D1 protein degradation. A serine protease requires specific amino acids to form a so-called charge relay system, normally involving an aspartic acid and a histidine interacting to stabilise the reactive γ-O on the catalytic serine [24]. This mechanism is independent of the substrate specificity. Once the proteolytic subunit of the photosystem II reaction centre has been identified, this present classification of the proteolytic activity will be important for elucidating the structural basis of D1 protein degradation using the sequence and structural information available [28].

At present, we have no explanation as to why the protease inhibitors do not give complete inhibition of the D1 protein degradation. It is possible that in the isolated photosystem II core complexes the catalytic site is partially shielded from the external phase, suggesting accessibility restrictions. Such restrictions are the most likely explanation for the lack of inhibition observed with intact thylakoid membranes, considering the shielded location of photosystem II in the lipid bilayer within the appressed regions of the grana stacks.

Apart from the conceptual significance with respect to the enzymatic mechanism of D1 protein degradation, our present observation on the effect of serine protease inhibitors should provide new experimental possibilities. It should be feasible, by addition of inhibitors, to trap an intermediate step in the photoinhibitory process where the D1 protein is damaged and triggered for proteolysis but not cleaved. Finally, addition of protease inhibitors may render photosystem II preparations more structurally stable, which should be of particular importance for attempts to crystallise the complex.

In agreement with our present observations it has very recently been shown [29] by using exogenous pep-

tide substrates that isolated photosystem II reaction centres possess an intrinsic serine protease activity.

Acknowledgements: This work was supported by the Swedish Natural Science Research Council. A.H.S. is the recipient of a post-doctoral fellowship from the Swedish Forestry and Agriculture Research Council. The antibodies were the kind gift of Dr. Wim Vermaas, Arizona State University. We acknowledge fruitful discussions with Drs. Anders Liljas and Cecilia Sundby, Chemical Center, University of Lund.

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