

Determination of Catabolism of the Photosystem II D1 Subunit by Structural Motifs in the Polypeptide Sequence

Catherine A. Shipton, Jonathan B. Marder*, and James Barber

AFRC Photosynthesis Research Group, Department of Biochemistry, Imperial College, London SW 7 2AY, U.K.

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Proteolytic mapping of the D1 subunit of photosystem two and a degradation product which arises during its rapid catabolism shows that the latter is a result of proteolysis within the peptide motif QEEET. This motif is located in a portion of the D1 protein thought to form a stroma-exposed connection between fourth and fifth transmembrane segments. This connection domain also contains a "PEST"-like sequence and forms part of the Q_B/herbicide binding niche. The QEEET motif seems to provide a major epitope in immunological studies, as judged from reaction of D1 and its fragments with polyclonal antibodies. Antibodies against D1 were found to react with other animal and plant proteins which contain similar sequence motifs.

Introduction

Rapid metabolism of the D1 protein was recognized some years ago (reviewed in [1]) before the central role of this polypeptide in the PS II reaction centre was appreciated. D1 is turned over at least an order of magnitude faster than the other polypeptide subunits of PS II and other major chloroplast proteins [1].

The mechanism of D1 degradation remains to be elucidated. It is a light-dependent process responding to wavelengths from the ultra-violet through the visible spectrum to above 700 nm [2, 3]. This wide spectral response indicates that the photochemical reactions which initiate D1 protein catabolism are at least partially distinct from the photosynthetic light reactions of PS II which show a narrower spectral response. It is not known whether the light dependence of degradation is due to effects on the conformation of the substrate D1 polypeptide, or due to activation of the proteolytic machinery.

Abbreviations: D1, D1 polypeptide subunit (*psbA* gene product) of photosystem two; D2, D2 polypeptide subunit (*psbD* gene product) of photosystem two; PS II, photosystem two; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; CHI, cycloheximide; Q_B, secondary acceptor quinone of PS II.

* Present address: Dept. of Horticulture, Hebrew University Faculty of Agriculture, Rehovot 76100, Israel.

Reprint requests to C. A. Shipton.

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Greenberg *et al.* [4] have recently produced evidence that catabolism of D1 proceeds *via* cleavage of the mature 32 kDa polypeptide to a 23.5 kDa fragment (apparent molecular mass by SDS-PAGE), the latter comprising the amino-terminal two thirds (approx.) of D1.

By analogy with bacterial reaction centres, the main apoproteins of the PS II reaction centre (D1 and D2) are thought to be organized in the thylakoid with five transmembrane alpha-helices in each polypeptide [5, 6]. In the case of D1 there is some experimental evidence for this arrangement [7]. The size of the 23.5 kDa breakdown product implies that it arises by cleavage of the stroma-exposed connection between the fourth and fifth transmembrane spans. This region is thought to form the binding site for Q_B (the secondary plastoquinone acceptor of PS II) and a number of PS II-inhibiting herbicides [8]. A diagram of this section of D1 is presented in Fig. 1, which shows the amino-acid sequence and depicts the suggested secondary structure. Greenberg *et al.* [4] noted a similarity between the sequence bounded by residues 225 and 238 and the "PEST" regions found in many other rapidly-degraded proteins [9]. "PEST" regions are so named because they are rich in proline (P), glutamate (E), serine (S), threonine (T) and other amino-acid residues which negatively influence any tendency to form alpha-helices. In Fig. 1 we prefer to designate this region as an "EST" sequence because of the absence of proline, although in other respects the D1 se-



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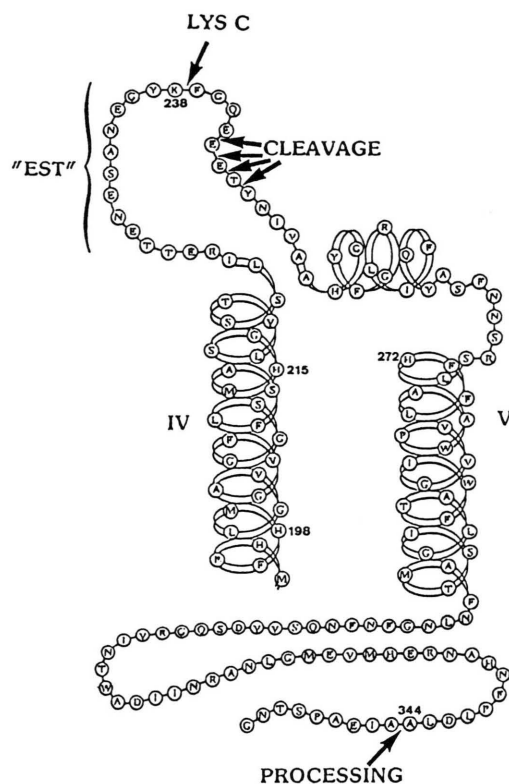


Fig. 1. Diagrammatic representation of a portion of the D1 protein. The diagram depicts the primary and secondary structure of a stroma-exposed portion of the protein as discussed in the text, and also shows the luminal-exposed C-terminus. Features discussed in the text are annotated on the proposed structure. These are the "EST" sequence, LYS C — a useful position for making specific proteolytic cleavages (in species containing a lysine residue at this position), and the CLEAVAGE site(s) responsible for *in vivo* D1 protein degradation. Also shown is the PROCESSING site which is cleaved during post-translational maturation of the D1 precursor.

quence between residues 225 and 238 qualifies as a "PEST" region. However, whether this "EST" region in the D1 protein contributes in any way to the rapid turnover rate is unknown.

Production of the 23.5 kDa breakdown product of D1 would appear to involve cleavage slightly to the carboxy side of residue 238 [4]. We now report the results of experiments which locate the cleavage site more precisely, and we show further that the cleavage region is important in determining the antigenic properties of D1.

Materials and Methods

Plant Material: Peas, wheat and barley were grown in vermiculite in a glasshouse maintained at 20–25 °C. *Lemna minor* was grown phototrophically in axenic culture [10], at room temperature under a 15 h photoperiod with illumination of 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ from cool-white fluorescent tubes.

Purified proteins: Human blood coagulation factor XIII and chick troponin were purchased from Calbiochem and Sigma respectively. Pea legumin and vicilin were a gift from Dr. R. Casey of the John Innes Institute. Poly-L-glutamate and poly-D-glutamate were purchased from Sigma.

Radioactive pulse labelling: *Lemna* and wheat plants were pulse-labelled with [^{35}S]methionine using procedures described elsewhere [11, 12].

Thylakoid isolation and protease treatment: Thylakoids were isolated using standard procedures [11, 13]. Enzyme digestions were performed on resuspended thylakoids [1–2 mg ml^{-1} chlorophyll] using endoproteinase Lys-C (Boehringer) [11] at 100 $\mu\text{g ml}^{-1}$.

SDS-PAGE and fluorography: Samples were analyzed on 7–17% polyacrylamide gradient gels as described elsewhere [11]. Radioactive samples were visualized by fluorography of dried diphenyl-oxazole-impregnated gels.

Immunoblotting: Profiles of separated proteins were electrophoretically transferred from polyacrylamide gels onto nitrocellulose and detected as described previously [14] using rabbit primary antibodies, biotinylated goat *anti*-rabbit immunoglobulin second antibodies (Sigma), extravidin-alkaline phosphatase conjugate (Sigma) and the appropriate chromogenic substrates. Primary antisera against D1 and D2 were raised using fusion proteins expressed from pUR vectors engineered to contain the *psbA* and *psbD* genes [15]. Where indicated in the figure legend, we have used an alternative antiserum against D1 [16] donated by Dr. J. Hirschberg of the Hebrew University. Affinity purified antibodies raised against purified pea legumin and vicilin were provided by Dr. R. Casey of the John Innes Institute. Antiserum against porcine pancreastatin was provided by Dr. D. Bretherton-Watt of the Royal Postgraduate Medical School. Antibodies against human blood coagulation factor XIII were purchased from Calbiochem. In some experiments (indicated in figure legends),

Immobilon (Millipore) was used instead of nitrocellulose; in these cases the total transferred protein profile could also be visualized by staining (as for gels) with Coomassie blue.

Computer analysis of sequence data: Searches for specific amino-acid sub-sequences were performed against a protein sequence database (SWISS-PROT Release 9, EMBL Data Library, December 1988) using the PC-GENE software package on a personal computer. The software was also used to predict antigenic regions in protein sequences by the method of Hopp and Woods [17].

Results

We have used the radioactive pulse-labelling conditions described previously for *Spirodela* [4] to visualize the 23.5 kDa breakdown fragment of D1 produced *in vivo* in *Lemna minor*. This is shown in Fig. 2.

In order to assess accurately the relative molecular mass of the breakdown fragment, we have compared it to a known 23 kDa "marker" frag-

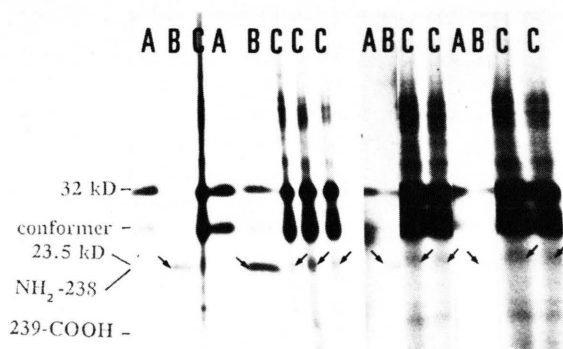


Fig. 2. Size determination of the *in vivo* breakdown product. Wheat leaves and *Lemna minor* fronds were radioactively pulse-labelled as described in Materials and Methods. For *Lemna*, labelling was done on medium containing CHI to enhance the appearance of the 23.5 kDa breakdown product [4]. Thylakoids were prepared and in some samples cleaved with Lys-C. The samples were analyzed by SDS-PAGE and fluorography. Lanes A show undigested wheat thylakoids. Lanes B show Lys-C digested wheat thylakoids. Lanes C show undigested thylakoids of *Lemna minor* fronds. Mobilities of D1-related bands are indicated on the left. The presence of 23.5 kDa *in vivo* breakdown product is shown by left-pointing arrows, and of the 23 kDa Lys-C generated marker fragment (NH₂-238) by right-pointing arrows.

ment of D1 which was produced *in vitro* by specific proteolysis at a unique lysine residue. This lysine occurs at position 238 in wheat D1 [12]; the marker fragment, is therefore known to consist of D1 residues from the amino-terminus up to and including residue 238. Fig. 2 shows that the marker fragment is slightly smaller than the 23.5 kDa breakdown product from *in vivo* degradation. By observing this mobility difference on several gels, and calculating the difference in molecular mass, we conclude that the breakdown product must extend from the amino-terminus beyond position 238 by 5–8 residues, which would arise from cleavage at any of the points indicated in Fig. 1. This is well within the cleavage region suggested by Greenberg *et al.* [4].

The sequence of the cleavage domain is notable for the presence of three consecutive glutamate residues. One possibility is that cleavage arises by proteolysis at one of these positions. We previously reported [18] attempts to emulate such a cleavage by using the V8 protease from *Staphylococcus aureus* which is specific for the carboxy-side peptide bond of glutamate residues. A map for V8 partial proteolysis is shown in Fig. 3. Cleavage of D1 at site 'B' would give a fragment of correct size. However, this fragment could only be obtained in small yield [18] due to the presence of a preferential V8 cleavage site 'A' which gives a 21 kDa fragment (Sa21). Nevertheless, the 23.5 kDa V8-generated band reacted relatively strongly with antiserum against D1 [18]. This taken together with the stronger immune-reaction of the smaller Lys-C digestion product relative to that of the larger fragment [12] led to the conclu-

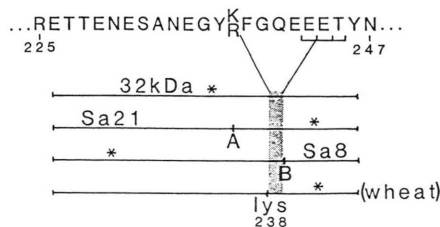


Fig. 3. Schematic representation of the D1 proteolytic map and location of the major epitope. Fragments showing strong immune reactions against D1 antiserum are marked with asterisks. The cleavage sites and fragment designations are discussed in the text and elsewhere [18]. The major epitope within D1 is deduced to lie within the shaded portion.

sion that the antiserum used contains a predominant population of paratopes which recognize the portion of D1 from residue 239 to residue 244, as summarized in Fig. 3.

We have used a computer algorithm to predict antigenic regions in the D1 sequence as described in Materials and Methods. The sequences from residues 6-11 (ERRDSE), 240-245 (GQEEET), and 225-231 (RETTENE) emerged as likely antigenic determinants. It seems very likely that the second of these regions is responsible for the reactions of the D1 antiserum described above. This sort of glutamate-rich sub-sequence occurs in a number [18], we predicted that the antiserum would perhaps react with some of these proteins. Figs. 4 and 5 show that the antiserum does indeed recognize other proteins, notably pea vicilin, a subunit of chick troponin, and human blood coagulation factor XIII. It should be noted that the major stainable band in the factor XIII lane is human serum albumin which is a major constituent of the commercial product. We do not believe that these cross-reactions are artefactual since a D1 antise-

rum prepared in another laboratory using an alternative bacterial vector for expressing the gene for D1 [16], also reacted with these proteins albeit to a much lesser degree (data for vicilin not shown). Table I summarizes the reactions, cross-reactions and non-reactions which we have observed between different proteins and antisera. The table also shows the sequence homologies to the D1 sub-sequence GQEEET. From the table, it is clear that a cluster of glutamic acid residues in a protein is insufficient to give a clear immune reaction with D1 antiserum. However, we have included in the table a very weak reaction (found by "dot blots") of D1 antiserum against poly-L-glutamate compared with no detectable reaction against poly-D-glutamate.

In view of the strong cross-reaction of the D1 antiserum with pea vicilin we considered the alarming possibility that we might be observing vicilin contamination in some pea thylakoid preparations. Indeed, we have in the past observed various cross-reacting bands which we were unable to identify [12, 14]. Fig. 5 shows an immunoblot of D1 antiserum against vicilin and various thylakoid preparations. We have used both pea and barley thylakoids, the latter serving as a control because this species contains no vicilin or closely related protein. There are three types of polypeptide band recognized by the visualization system used. The first type is represented by the 74 kDa band of pea thylakoids, which appears using all antibodies (compare anti-vicilin and anti-D1 reactions in Fig. 5) or even with no antibody (not shown). The appearance of this band is therefore artefactual and probably due to biotinylation, causing the protein to bind the avidin-conjugated enzyme used for visualization. The second type of reaction detects genuine D1-related bands. In Fig. 5, these are the D1 32 kDa band and 28 kDa conformer from pea and barley thylakoids, and the 23 kDa and 11 kDa D1 fragments from lysine-specific proteolysis of barley thylakoids (N.B. barley, like wheat, contains a lysine residue at position 238 [19]). The third type of reaction is due to paratopes in the D1 antiserum which recognize proteins unrelated to D1. In Fig. 5, the cross-reaction against constituent polypeptides of vicilin is obviously of this type. We also observe cross-reacting bands of 24.5 kDa and 15 kDa (approx.) which have only ever been observed in thylakoids of pea. Unlike pea D1, both these bands are fully digesti-

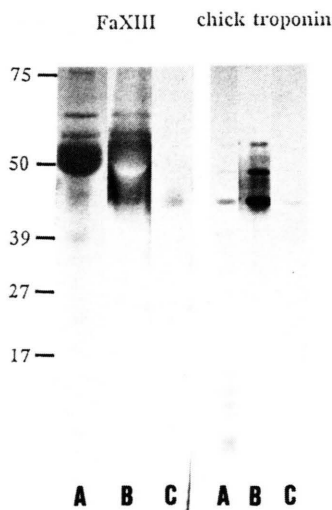


Fig. 4. Reactions of D1 antiserum against two animal proteins. Electrophoretically separated proteins, human blood coagulation factor XIII (FaXIII) and chick troponin, were blotted onto Immobilon membrane. Lanes A were stained with Coomassie blue. Lanes B show bands which react with a D1 antiserum produced in our laboratory [15]. Lanes C show bands which react with antiserum to D1 produced in another laboratory as reported in [16].

Table I. Reactions and cross reactions of antibodies against D1 and other proteins containing homologies to the D1 cleavage site.

Polypeptide Protein	Gene	Sequence	Antibodies against					
			D1	D2	Vicilin	Legumin	Panc.	FaXIII
D1	PSBA	^R FGQEEETYN ¹	***	—	—	—	—	—
D2	PSBD	nptQaEETYN	—	***	—	—	—	—
Vicilin	VCLB	eeeQEEETsk	***	—	***	* ²	n.d.	n.d.
Legumin	LEGA	hhqQEE Esee	—	—	* ³	***	n.d.	n.d.
Panc.	PCST	hsrQEE Eeet	*	—	n.d.	n.d.	n.d.	n.d.
FaXIII	F13A	qeGQEE Erla	**	n.d.	n.d.	n.d.	n.d.	n.d.
Trop. T	TRTC	eeeQEE Eyyve	**	n.d.	n.d.	n.d.	n.d.	n.d.
poly-L-Glu	—	eeee EEEEEe	*	n.d.	n.d.	n.d.	n.d.	n.d.
poly-D-Glu	—	eeee EEEEEe	—	n.d.	n.d.	n.d.	n.d.	n.d.

*** Strong reaction

** Moderate reaction

* Weak reaction

— No detectable reaction

n.d. Not determined

Antibodies and proteins used are described in Materials and Methods and are designated in the table as follows: D1 (band in solubilized total thylakoids), D2 (band in solubilized total thylakoids), Vicilin (purified from pea seeds), Legumin (purified from pea seeds), Panc. (Porcine pancreastatin), FaXIII (human blood coagulation factor XIII), Trop. T (T subunit of chick troponin), poly-L-Glu (homopolymer of L isomer of glutamic acid), poly-D-Glu (homopolymer of D isomer of glutamic acid).

Gene names and sequences are derived from SWISS-PROT database (see Materials and Methods).

¹ The D1 polypeptide contains either arginine or lysine at position 238 depending on species (see text).

² Reaction probably due to vicilin contamination in legumin preparation.

³ Reaction probably due to legumin contamination in vicilin preparation.

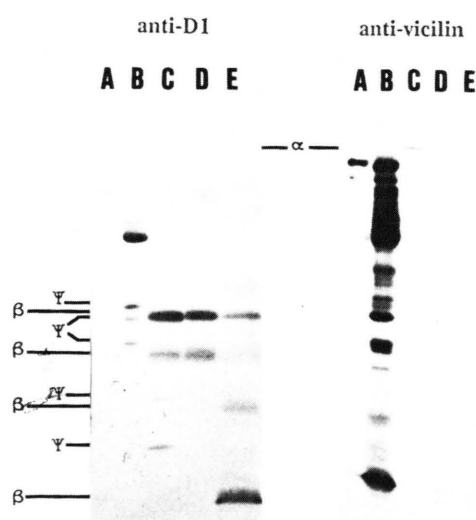


Fig. 5. Immunoblot showing three reaction mechanisms of the D1 antiserum. Thylakoids of pea and barley were prepared and a sample of barley membranes cleaved with Lys-C. Proteins were separated, blotted and visualized as described in Materials and Methods. Lanes A and B show the pea storage proteins legumin and vicilin respectively. Lanes C show undigested pea thylakoids. Lanes D show undigested barley thylakoids. Lanes E show Lys-C digested barley thylakoids. Bands indicated α are artefactual (see text). Bands marked β are genuine D1-related proteins. Ψ indicates polypeptide bands unrelated to D1 which nevertheless react with the antiserum against D1.

ble by lysine-specific proteolysis (not shown); lysine-specific digestion of the 15 kDa band has been shown previously (Fig. 1 in ref. [12]). Neither of these two bands are equal in size to any of the bands observed in Fig. 5 for the vicilin preparation. However, vicilin polypeptides of this molecular weight can arise by post-translational cleavage [20]. We have considered the possibility that the 15 kDa and 24.5 kDa bands might be vicilin fragments which arise by interaction of vicilin with pea thylakoids, but in the absence of a clear immune reaction of these bands with the vicilin antibodies available for this work, we are unable to verify this assignment. Neither can we dismiss the possibility since the vicilin antibodies may not themselves recognize the QEEET sub-sequence (note lack of anti-vicilin reaction with D1 in Fig. 5), and there are several polypeptides in the vicilin preparation which also do not react with vicilin antibodies.

Discussion

We have shown that a sub-sequence of D1 forms a target both for *in vivo* proteolysis during D1 catabolism, and for eliciting an antibody response. It is not unreasonable to envisage that this sub-sequence forms a strong motif of secondary structure which is recognized both by a chloroplast protease, and also by the mammalian immune system. The sequence is distinguished by being extremely rich in acidic amino-acid residues. Although the presence of this sequence distinguishes D1 from other less-rapidly catabolized chloroplast proteins, similar sequences are found in a number of diverse non-plastid-related proteins. These include examples of proteins known to display rapid catabolism *e.g.* cytochrome P450 [21], or expected to show rapid catabolism by virtue of their physiological function *e.g.* mammalian hormones chro-

mogranin, pancreastatin and calcitonin. Plant storage proteins such as vicilin and legumin undergo rapid mobilization during seed germination, which is thought to involve digestion in the polyglutamate stretches (R. Casey, personal communication).

We do not wish to speculate on how a seed protein might come to reside in leaf tissue, or on how it might come into contact with internal chloroplast membranes (though this latter interaction may only occur during thylakoid isolation). Nevertheless, it is fascinating to speculate on the possible presence in thylakoids of a protease which recognizes the sequence QEEET in both D1 and vicilin! With this in mind, we have performed incubations of vicilin with wheat and barley thylakoids, but have not so far been able to detect any digestion products of vicilin. We plan to test other foreign proteins besides vicilin, and other incubation conditions including perhaps illumination in order to develop a new assay for the proteolytic machinery of D1 catabolism.

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