

Characterization of Conformers of D 1 of Photosystem II Using Site-Directed Antibodies

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D1 from its resistance (pea) or susceptibility (wheat) to lysine-C degradation. A monoclonal

Antibodies have been raised to synthetic peptides, corresponding to a region in the loop spanning helices 4 and 5 of D1 protein (Ala 250–Phe 265) and to a region anticipated to be near the C terminus of mature D1 (His 332–Ala 345). Polyclonal antibodies to the sequence His 332–Ala 345 reacted with a 32 kDa polypeptide in thylakoid preparations identified as D1 from its resistance (pea) or susceptibility (wheat) to lysine-C degradation. A monoclonal antibody to His 332–Ala 345 reacted preferentially with a faster migrating polypeptide in SDS electrophoresis, a putative conformer of D1. Polyclonal antibodies to the sequence Ala 250–Phe 265 also reacted with the faster running polypeptide but not with the population of molecules running at 32 kDa. The putative conformer of D1 from wheat appears to be more resistant than the main D1 population to lysine-C degradation. Peptide analyses by Takahashi *et al.* [(1988) FEBS Lett, 240, 6–8] suggest Asn 335–Ala 344 lies at the processed C terminus. The present report provides immunological confirmation that this sequence is retained in mature D1.

Introduction

The reaction centre of photosystem II is probably a heterodimer of D1 and D2 proteins analogous to the L and M subunits of the bacterial reaction centre [1–4]. The amino acid sequence of the 32 kDa D1, herbicide-binding protein is highly conserved as deduced from DNA sequence analysis [5]. This polypeptide is synthesized on thylakoid-bound ribosomes as a precursor 1–2 kDa larger than the mature polypeptide, prior to being incorporated into the PS II reaction centre in the 32 kDa form [6]. Using peptide mapping, Marder *et al.* [7] proposed that the C terminus is processed with the loss of about 12–16 amino acids. Subsequently, it was shown that polyclonal antibodies to the 14 amino acids of the C terminus of precursor D1 (Pro 340–Gly 353), predicted from the

psbA gene nucleotide sequence of spinach, cross reacted only with D1 from a mutant alga which fails to process pre-D1 [8] and not with mature D1 from spinach or the wild alga. These observations indicate that this 14 amino acid epitope was removed during processing. However, subsequent amino acid sequencing of D1 peptide fragments from D1 of spinach thylakoids revealed the sequence Asn 335–Ala 344, indicating only 9 amino acids may be cleaved from the C terminus of precursor D1 during maturation [9]. In this report immunological evidence is presented confirming this sequence is retained in mature D1. Further properties of the putative conformer are also presented.

Materials and Methods

Peptide synthesis and crosslinking

The peptides were synthesized on a Biosearch Model 9500 peptide synthesizer by a solid phase method [10]. Cysteine was added to the C-terminal end of the tetradecapeptide HERNAHNFPLDLAA, corresponding to deduced amino acid sequence His 332–Ala 345 of D1, and to the N-terminal end of the hexadecapeptide AAHGYFGRLIFQYASF, corresponding to a deduced region Ala 250–Phe 265 of D1 for coupling to PPD (purified protein derivative of

Abbreviations: BCG, Bacillus Calmette-Guerin; ELISA, Enzyme linked immunosorbent assay; PBS, phosphate buffered saline; HRP, Horseradish peroxidase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Sulpho SMCC, Sulpho-succinimidyl 4-(N-Maleimido-methyl)cyclohexane-1-carboxylate; PPD, purified protein derivative of tuberculin; PAR, photosynthetically active radiation.

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tuberculin). To 15 mg PPD (Statens Serum Institut, Copenhagen) in 2.3 ml PBS was added 7.5 mg sulpho-SMCC (Pierce U.K. Ltd.) in 1 ml PBS and the mixture incubated at room temperature for 1 h. After 1 h the conjugate was separated from unreacted crosslinker by gel filtration on a G-25 column (void volume 8 ml) using PBS buffer. To one third of the eluate collected between 8 ml and 14.3 ml was added 5 mg of peptide in 1 ml PBS, stirred at room temperature for 2 h. The carrier-crosslinker-peptide was passed through a Sephadex G-25 column using PBS. The tetradecapeptide RAAEDPEFETFYTK corresponding to the deduced conserved sequence Arg 305–Lys 318 in D2 was coupled to PPD *via* the tyrosine groups present in both molecules. First *o*-toluidine was diazotized using 0.023 g *o*-toluidine HCl in 4.5 ml 0.2 M HCl to which was added 0.0175 g NaNO₂ in 0.5 ml H₂O at 4 °C and stirred for 1 h. To 5 mg PPD in 1 ml 0.16 M Na borate buffered saline, pH 9.0 and 5 mg peptide in 1 ml borate on ice was added 0.6 ml of the bis-diazotized *o*-toluidine. The mixture was adjusted to pH 7.4 with 0.1 N NaOH and kept on ice for 2 h in the dark with occasional stirring. Low m.wt. toxic products were removed using a Sephadex G-25 column eluted with PBS.

Antibody production

Four female rats were primed with BCG and subsequently immunized with 100 µg of PPD-peptide His 332–Ala 345 conjugate in incomplete Freund's adjuvant. After two immunizations rats were tested for antibodies to the peptide. The rat with the highest titre was boosted intravenously with 50 µg of conjugate in saline and 3 days later the spleen cells were used for fusion. Cell lines secreting anti D1 peptide were derived from fusions of spleen cells with plasmacytoma 653 and cloned by limiting dilution as described in Galfre and Milstein [11]. Monoclonal antibodies were classified by Ouchterlony analysis using class specific antisera. Polyclonal antibodies were also raised in rabbits to PPD conjugated to D1 sequences His 332–Ala 345 or Ala 250–Phe 265 and to PPD conjugated to D2 sequence Arg 305–Lys 318.

Screening of sera and culture supernatants

Rat and rabbit sera, and culture supernatants were screened for antipeptide antibodies using free

peptides and indirect ELISA. 50 µl of peptide (10 µg ml⁻¹) in PBS were added to 96-well microtitre plates (Dynatech Ltd., U.K.) and incubated for at least 2 h at room temperature. Wells were blocked for at least 2 h with 150 µl of 1% (w/v) BSA in PBS. After removing blocking solution 50 µl of test antibody appropriately diluted in PBS–BSA containing 0.05% (v/v) Tween 20 were added for 2 h at room temperature. Plates were washed three times with PBS–Tween and either rabbit anti rat-HRP conjugate (ICN Biomedicals Ltd., U.K.) or goat antirabbit – HRP conjugate (Sigma Ltd., U.K.), diluted 1:1000 in PBS–Tween–BSA was added for 2 h at room temperature. After washing, 100 µl of peroxidase substrate (150 µl of 10 mg ml⁻¹ 3, 3', 5, 5' tetramethylbenzidine in dimethyl sulphoxide, 15 µl of 6% (w/v) H₂O₂, 1.5 ml 1 M Na acetate-citric acid, pH 6 and 13.5 ml H₂O) was added to each well. The reaction was stopped by adding 20 µl of 2 M H₂SO₄ and the absorbance read at 450 nm using a Bio-Rad model 2550 EIA Reader.

SDS-PAGE and immunostaining of thylakoid preparations

Crude thylakoid preparations were made essentially as described in [12]. Leaf tissue (0.2 g) from lettuce, pea or wheat was frozen in liquid N₂ powdered by means of a pestle and mortar and further homogenized using 0.8 ml of 50 mM Tris-HCl (pH 8.0) buffer containing 0.2 M sucrose, 10 mM NaCl, 0.1% (w/v) NaN₃ and 1 mM phenyl methylsulphonyl fluoride. The homogenate was filtered through two layers of Miracloth and the filtrate centrifuged for 5 min at 15,000 × *g*. The pellet fraction was solubilized in 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.4 for 5 min in boiling water. The SDS-treated proteins (containing up to 10 µg chlorophyll equivalent) were subjected to electrophoresis on a discontinuous gradient of SDS-polyacrylamide consisting of a 10% (w/v) acrylamide resolving gel and a 3% (w/v) stacking gel using a Hoefer minigel SE 200. Western blotting and immunostaining were as described in [13].

Proteolysis of thylakoids

Crude thylakoids prepared as above were suspended in 50 mM Tris-HCl (pH 8.0) buffer, 10 mM

KCl, 10 mM MgCl₂ and incubated in the absence or presence of Lys-C endoproteinase (Boehringer) added to 0.1 or 1 mg ml⁻¹ for 4 h at room temperature in the dark. Subsequently samples were solubilized in 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 50 mM Tris-HCl pH 7.4 for 5 min in boiling water. Peptide fragments were identified on a 12.5% (w/v) acrylamide gel.

Results and Discussion

Examination of the amino acid sequence of D1 deduced from the *psbA* gene nucleotide sequence from a wide range of species shows a highly conserved region which might be close to the new C terminus formed after C terminal processing [9]. The region His 332–Ala 345 has a high hydrophilic score [14] and is predicted to be a good antigenic site [15]. A second site was chosen, Ala 250–Phe 265, which forms part of the loop thought to connect the transmembrane helices 4 and 5. There is evidence that this loop binds either the plastoqui-

none Q_B or herbicides and is a mutation site (*e.g.* Ser 264) conferring herbicide resistance. Just before the sequence Ala 250–Phe 265 towards the N terminus is the suggested cleavage point for protein turnover [16]. The sequence Ala 250–Phe 265 is highly conserved across species but has a lower hydrophilic and antigenic score than the C terminal sequence. For immunizations, PPD was used as the carrier protein since antibodies are rarely produced against it but it generates strong T-cell response in BCG-sensitized rats [17].

The polyclonal antibodies raised to His 332–Ala 345 bind to a polypeptide of approximately 32 kDa, corresponding to D1 in SDS-treated crude thylakoid preparations from pea (Fig. 1A), lettuce and wheat. There is also an immunoreactive band which migrates just above 49 kDa. A reactive band (or bands) of similar high m.wt. has been observed previously [16, 18, 19] and has been suggested to be aggregates of D1 or possibly a heterodimer containing D2 (D1–D2). A band of similar m.wt. is detected when challenged

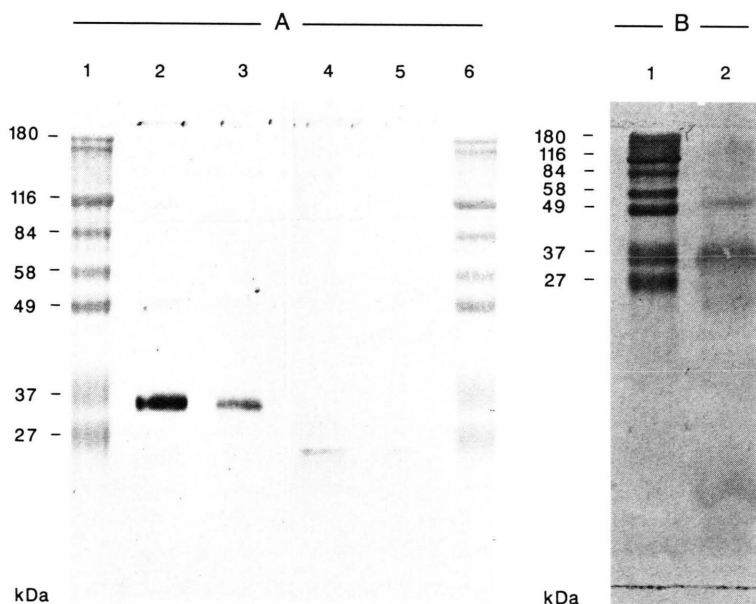


Fig. 1. Electrophoretograms of pea thylakoids after SDS-PAGE probed with antibodies to tetradecapeptides synthesized either from the deduced D1 sequence His 332–Ala 345 or from the deduced D2 sequence Arg 305–Lys 318. (A) Lanes 1 and 6, molecular mass markers; lanes 2 and 3, thylakoid loading equivalent to 1.2 µg or 0.6 µg chlorophyll, respectively, probed with polyclonal antibodies raised in a rabbit to His 332–Ala 345 deduced from the *psbA* gene of pea and spinach; lanes 4 and 5, thylakoid loading equivalent to 1.2 µg or 0.6 µg chlorophyll, respectively, probed with monoclonal antibodies to deduced D1 sequence His 332–Ala 345. (B) Lane 1, molecular mass markers; lane 2, thylakoid loading equivalent to 10 µg chlorophyll, probed with polyclonal antibodies raised in a rabbit to a tetradecapeptide Arg 305–Lys 318 deduced from the *psbD* gene of pea and spinach.

with antibodies directed to a sequence in D2 (Fig. 1 B) and multiple bands with polyclonals to the Ala 250–Phe 265 sequence in D1 (Fig. 2A). On longer incubations with polyclonals to D1 His 332–Ala 345 another band develops with a molecular mass approximately 26 kDa (not shown). A monoclonal antibody directed to the D1 sequence His 332–Ala 345 binds to a faster migrating polypeptide at approximately 26 kDa but does not bind to the 32 kDa band (Fig. 1A). It has been suggested this faster running band is a conformer of D1 [20, 21].

Additional evidence that the faster moving species is D1 is that the molecule from lettuce is recognized by polyclonal antibodies raised to the sequence Ala 250–Phe 265 (the loop connecting helices 4 and 5 (Fig. 2A)) and by monoclonal antibodies to His 332–Ala 345 (Fig. 2B). Recognition of this band by both site-directed antibodies which have different specificity provides direct evidence

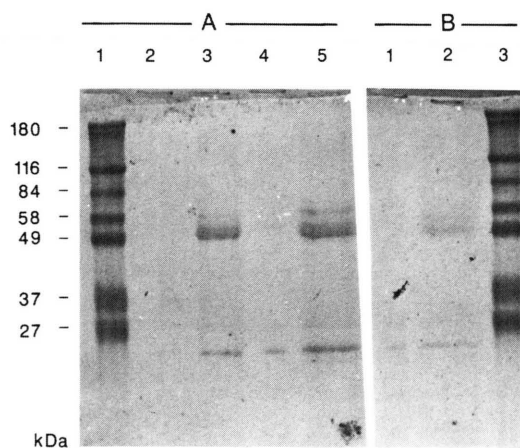


Fig. 2. Electrophoretograms of lettuce thylakoids probed with antibodies directed to the deduced D1 sequence Ala 250–Phe 265 or His 332–Ala 345. (A) Lane 1, molecular mass markers; lanes 2 and 4, low-light grown ($100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, PAR) lettuce thylakoids loading 2.4 μg or 4.5 μg chlorophyll; lanes 3 and 5, high-light grown ($400 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, PAR) lettuce thylakoids loading 3.2 μg or 6.4 μg chlorophyll, probed with rabbit polyclonal antibodies elicited by a hexadecapeptide Ala 250–Phe 265 deduced from the *psbA* gene of pea and spinach corresponding to a region of a loop thought to connect helices 4 and 5 of D1. (B) Lanes 1 and 2, low- or high-light grown lettuce thylakoids loading 4.8 μg or 6.4 μg chlorophyll, respectively, probed with monoclonal antibodies to deduced D1 sequence His 332–Ala 345; lane 3, molecular mass markers.

that this band contains the two sequences common to D1. This polypeptide may have a more open structure than the main population of D1 at 32 kDa since it is attacked more readily by papain, but produces the same molecular weight peptide fragments as the main population of D1 [21].

A more open structure would be more accessible to antibody binding. SDS-treated proteins after electrophoresis lose most of the bound SDS on Western blotting. During subsequent incubation in PBS or PBS with blocking protein, considerable refolding of proteins can occur, hydrophobic domains attach to or embed within the nitrocellulose/nylon membrane and the hydrophilic regions remain in solution. The monoclonal antibody, which is an IgM, may be sterically hindered in attaching to the C terminal epitope in the polypeptide running at 32 kDa. It is suggested that the main form of D1, running at 32 kDa, refolds after Western blotting rendering the C terminus less accessible. The polyclonals can bind to this site presumably because they are a mixed population of immunoglobulins containing low m.wt. IgG.

For further evidence that the polyclonal antibodies to His 332–Ala 345 are binding to D1, thylakoid preparations were subjected to lysine-C proteolysis. From the amino acid composition deduced from nucleotide sequences wheat D1 contains one lysine at position 238 but this is replaced by arginine in pea [22, 23]. Fig. 3 shows that the polyclonal antibodies recognize a lysine-C resistant polypeptide in pea and lettuce. This indicates that lettuce D1 is also lysine-free. With wheat thylakoids the level of 32 kDa polypeptide decreases when incubated with lysine-C with a corresponding increase in degradation product at m.wt. approx. 11 kDa. This is consistent with clipping at Lys 238. An immunoreactive band(s) also appears running at approximately 45 kDa which may be the 11 kDa fragment binding to D1 and/or D2.

Using the monoclonal antibody to His 332–Ala 345 the faster running D1 species from wheat appears to be more resistant to lysine-C degradation (Fig. 4A). This antibody does not recognize the His 332–Ala 345 sequence in the main D1 polypeptide or in the 11 kDa fragments from the main D1 population after lysine-C treatment. Polyclonal antibodies which bind to the loop site Ala 250–Phe 265 of the faster running band also indicate that this band is relatively resistant to lysine-C

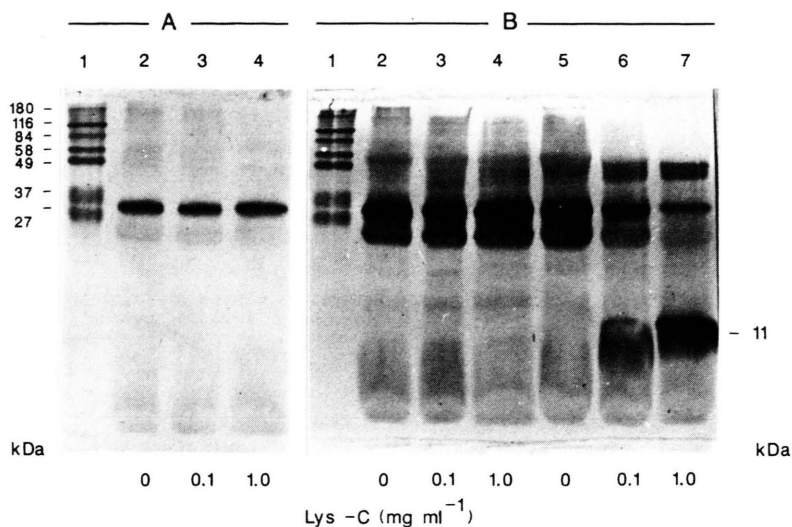


Fig. 3. Electrophorblotted polypeptides of pea, lettuce and wheat thylakoids after lysine-specific proteolysis probed with polyclonal antibodies to deduced D1 sequence His 332–Ala 345. (A) Lane 1, molecular mass markers; lanes 2, 3 and 4, pea thylakoids. (B) Lane 1, molecular mass markers; lanes 2, 3 and 4, lettuce thylakoids; lanes 5, 6 and 7, wheat thylakoids. Thylakoids incubated 4 h at room temperature in the dark with increasing concentrations of lysine-C endoproteinase. Loading equivalent to 10 μg chlorophyll.

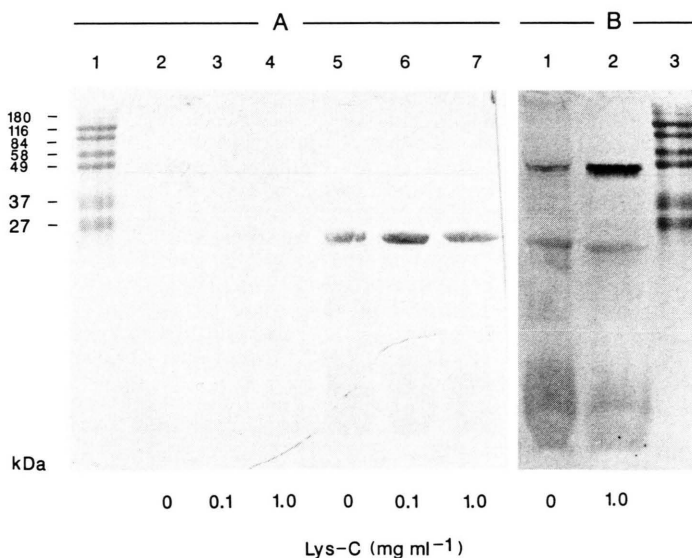


Fig. 4. Electrophorblotted polypeptides of wheat thylakoids after lysine-specific proteolysis probed with monoclonal antibodies to deduced D1 sequence His 332–Ala 345 or probed with polyclonal antibodies to part of loop sequence Ala 250–Phe 265 connecting helices 4 and 5 of D1. (A) Lane 1, molecular mass markers; lanes 2, 3 and 4 wheat thylakoids treated with increasing concentration of lysine-C and probed with pre-immunized rat serum; lanes 5, 6 and 7, wheat thylakoids treated with increasing concentrations of lysine-C and probed with monoclonal antibodies to His 332–Ala 345 in D1. (B) Lanes 1 and 2, wheat thylakoids incubated in absence or presence, respectively, of lysine-C and probed with polyclonal antibodies to loop sequence Ala 250–Phe 265 in D1; lane 3, molecular mass markers. Loading equivalent to 10 μg chlorophyll.

degradation (Fig. 4B). However, the 11 kDa fragment clipped from the main D1 population is detected when attached to a high molecular mass polypeptide with combined m.wt. approx. 45 kDa (Fig. 4B). It is suggested that the sequence Ala 250–Phe 265 in the free 11 kDa fragment is not sufficiently exposed when immobilized on nitrocellulose for binding to the polyclonal antibodies. However, when the fragment binds to a higher molecular mass species (see also Fig. 3) the sequence is then accessible to these site-directed polyclonal antibodies (Fig. 4B). The nature of the resistance of the faster migrating D1 to lysine-C degradation is yet to be elucidated.

Diner *et al.* [8] constructed a tetradecapeptide Pro 340–Gly 353 from the sequence of the unprocessed C terminal end of D1 and the polyclonal antibodies to it did not react with mature D1. Consequently, they suggested that at least 14 amino acids were removed during processing. However, peptide fragments of mature D1 have been

analyzed [9] and a sequence corresponding to the segment from Asn 335–Ala 344 was identified. This indicated that only 9 amino acids may be cleaved from the C terminus of pre D1 during maturation. In the present report antibodies to the epitope His 332–Ala 345 react with the 32 kDa polypeptide. Hence these antibodies recognize the sequence His 332–Ala 344 suggested from peptide analysis to be at the C terminus of mature D1. To the authors' knowledge this is the first immunological evidence that the sequence His 332–Ala 344 is retained in processed D1.

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