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The D1 polypeptide subunit of the Photosystem II reaction centre has a phosphorylation site at its amino terminus

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The D1 polypeptide subunit of the Photosystem II reaction centre was phosphorylated by incubating isolated pea and wheat thylakoids with radioactive ATP. The phosphate label was followed through defined enzymatic digestion procedures, using D1-specific antibodies and sequence information to detect and identify expected cleavages. The phosphorylation site was determined to be at the amino terminus of D1, most probably on the threonine residue at position 2.

Phosphorylated D1 has previously been demonstrated as a distinct 32 kDa band in Photosystem II reaction centres prepared from pea thylakoids which had been incubated with radioactive ATP under reducing conditions [1]. We report here on work carried out on wheat and pea thylakoids which were isolated and phosphorylated in the dark by incubating with [γ-^{32}P]ATP and sodium dithionite as previously described [1]. The thylakoid proteins were subjected to defined digestions with specific proteolytic enzymes and then analysed by SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose and immunoblotting with D1-specific antibodies as described elsewhere [2]. The phosphoproteins were visualised by autoradiography of the same nitrocellulose-fixed electrophoretic profiles. In Fig. 1, phosphorylated D1 appears as a distinct labelled band in pea and wheat thylakoids despite the presence of several other phosphorylated polypeptides. The band is identifiable as D1, since it coincides with the band on the gel reacting most strongly with specific antibodies to this protein (D1 blot). The cross-reaction of the antibody with other bands has been discussed previously [2]. It should be noted that the band at about 28 kDa is due to a population of fast-migrating D1 molecules rather than antibody cross-reaction with another protein. The D1 band was further identified by subjecting the thylakoids to lysine-specific proteolysis as described in the legend to Fig. 1. In pea thylakoids, the 32 kDa phosphoprotein (Fig. 1, pea, ^{32}P), like the antibody-reactive band (Fig. 1, pea, D1 blot), was totally resistant to digestion, as is expected from the absence of codons for lysine in the (*psb*A) gene sequence for pea D1 [3].

In wheat, the *psb*A gene is highly homologous indicating just five amino-acid differences between the two species over the entire 353-codon reading frame. Thus wheat D1 contains residues Glu-9, Thr-12, Lys-238, Val-346 and Val-348 [4] compared to Asp-9, Asn-12, Arg-238, Ile-346 and Ala-348 in pea. Fig. 1 shows that in contrast to pea D1, wheat D1 is susceptible to lysine-specific proteolysis. This is directly attributable to clea-

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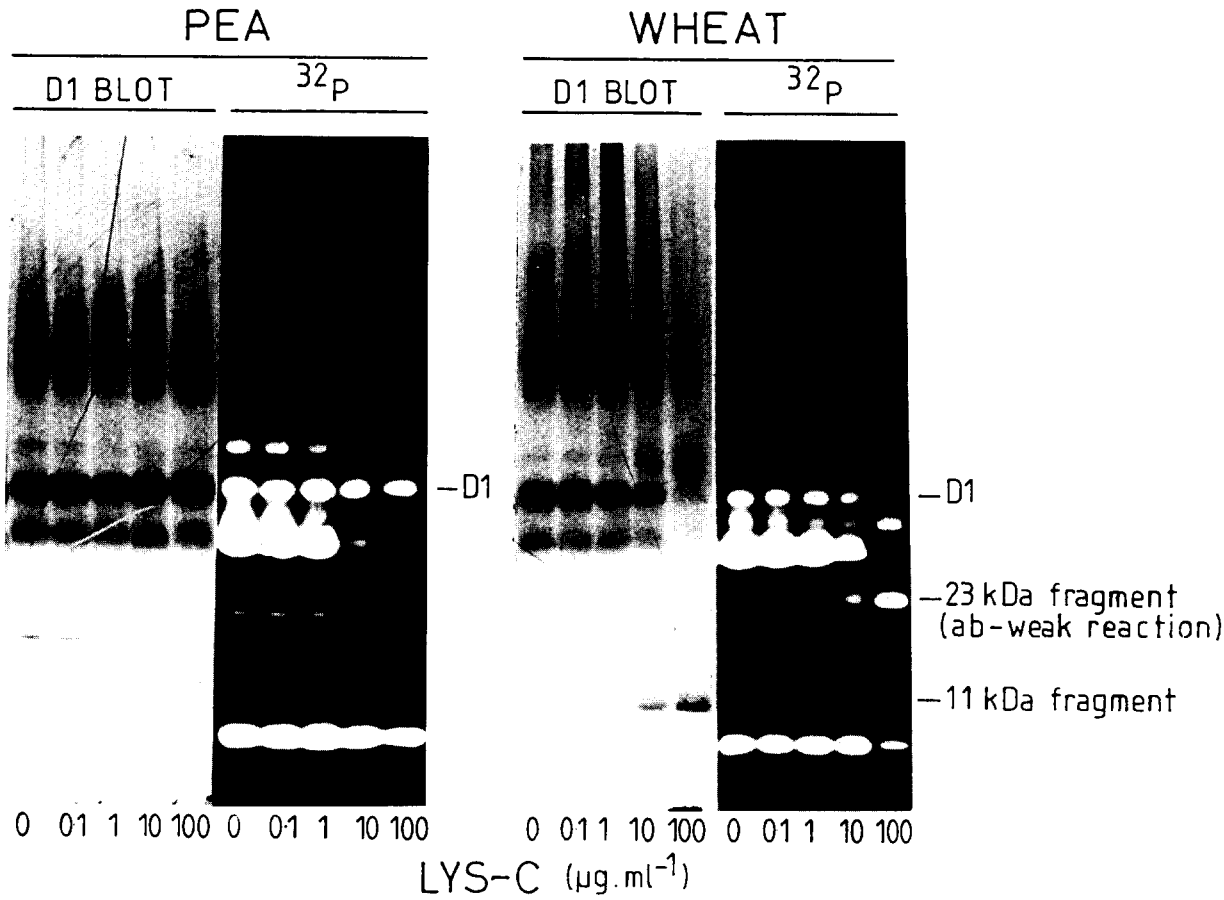

Fig. 1. Lysine-specific proteolysis of phosphorylated pea and wheat thylakoids. Thylakoids were isolated and radioactively phosphorylated as described elsewhere [1]. Digestion was performed by resuspending the thylakoids to 0.5 mg chlorophyll per ml in 0.33 M sorbitol, 10 mM KCl, 10 mM $MgCl_2$, 10 mM Tricine (pH 8.0) containing the indicated concentrations of Lys-C endoprotease (Boehringer). After incubation at room temperature for 1 h, the samples were fractionated by SDS-polyacrylamide electrophoresis on a 7–17% gel and transferred to nitrocellulose which was then probed with D1-specific antibodies [2]. The figure shows the antibody-reacting bands (D1 blot) and autoradiogram of the nitrocellulose (^{32}P).

vage at the unique lysine residue at position 238. Immunoblots with D1-specific antibodies show loss of the 32 kDa D1 band and appearance of an 11 kDa digestion product which presumably corresponds to the fragment to the carboxy side of Lys-238 (Fig. 1, wheat, D1 blot). However, the antibodies react very weakly to the 23 kDa amino-terminal fragment. The corresponding autoradiogram shows a phosphorylated digestion product at the latter molecular weight (Fig. 1, wheat, ^{32}P). Because of the poor antibody reaction, the identity of the 23 kDa band as a D1 fragment was further established using [^{35}S]methionine pulse-labelling as a specific probe for D1 [5]. The lysine-specific digestion of [^{35}S]methionine-labelled wheat D1 is shown in Fig. 2 (lanes ^{35}S) together with the digestion of phosphorylated wheat thylakoids (lanes ^{32}P). The ^{35}S label clearly identifies the 11 and 23 kDa D1 fragments, the latter migrating with the same apparent molecular weight as the 23 kDa phosphorylated digestion product. The unequal distribution of ^{35}S label between the 11 and 23 kDa fragments reflects the positions of the 12 methionine residue indicated by the gene sequence, i.e., nine methionines to the amino side of residue 238 and three methionines to the carboxy side. Thus the 23 kDa digestion product appears to consist of the D1 sequence

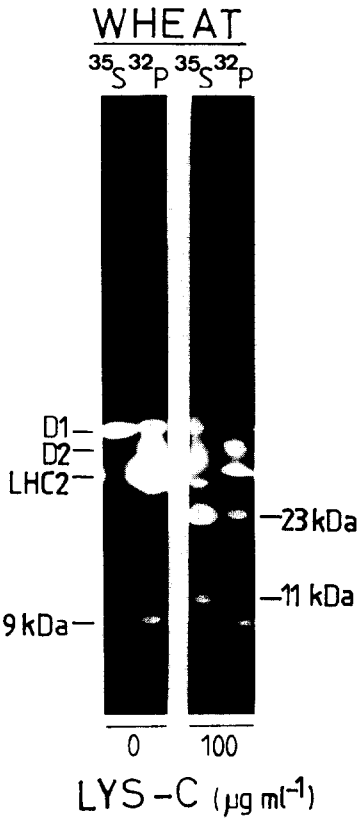

Fig. 2. Lysine-specific proteolysis of [^{35}S]methionine-labelled wheat thylakoids. Wheat leaf pieces were incubated with illumination for 3 h on a medium containing [^{35}S]methionine as described elsewhere [5]. Thylakoids were isolated, digested with Lys-C and analysed by electrophoresis as described in the legend to Fig. 1. Phosphorylated wheat thylakoids (Fig. 1) were exposed to the same digestions and analysed in adjacent gel lanes. The gel was then impregnated with diphenyloxazole and exposed for fluorography [5]. The resulting radioactive image arising from the [^{35}S]methionine label (lanes ^{35}S) and phosphate label (lanes ^{32}P) is shown in the figure. The positions of the main phosphorylated bands are identified to the left of the figure and of the two D1 fragments to the right.

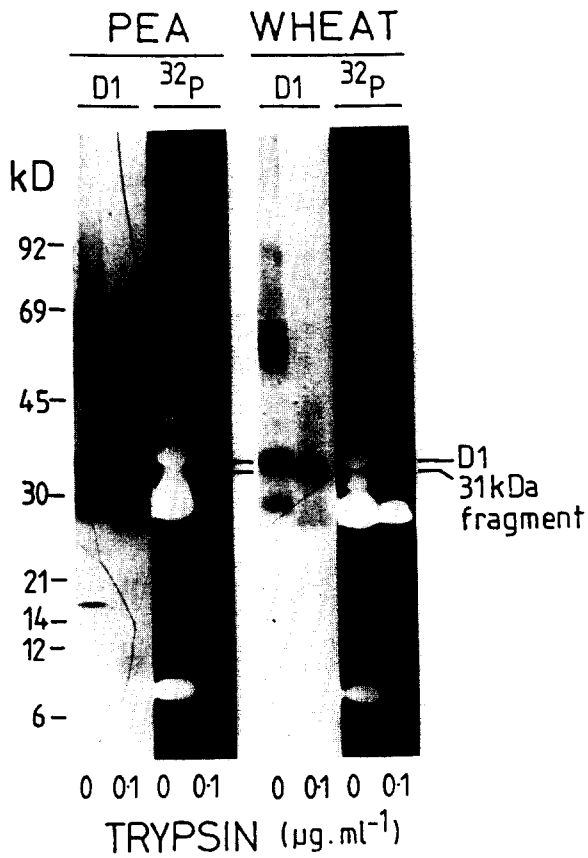

Fig. 3. Trypsinisation of phosphorylated pea and wheat thylakoids. Radioactively phosphorylated pea thylakoids and wheat thylakoids were incubated as in Fig. 1 for 1 h in the presence or absence of 0.1 μg of trypsin per ml to achieve digestion of the D1 polypeptide to a 31 kDa fragment [5,6]. The treated samples were analysed by electrophoresis, transfer to nitrocellulose, reaction with D1-specific antibodies (D1) and autoradiography (^{32}P) as in Fig. 1.

from the amino terminus to residue 238. It therefore follows that the phosphorylated fragment at 23 kDa consists of this same sequence, since it appears under similar cleavage conditions to the ^{35}S-labelled band and migrates with the same apparent molecular weight. These data therefore indicate that the phosphorylation site is located between the amino terminus and residue 238.

The location of the phosphorylation site in D1 was further studied by trypsinisation as described in the legend to Fig. 3. It was previously shown in *Spirodela* thylakoids that mild trypsin digestion removes a small fragment of D1 to yield a 31 kDa band designated T31 [6]. This was reproduced in thylakoids from phosphorylated pea and wheat, using D1-specific antibodies to follow the digestion (Fig. 3, D1). The cleavage must be due to removal of a few amino-acid residues from either or both termini of the protein. The autoradiogram (Fig. 3, ^{32}P) shows that T31 is not phosphorylated, presumably because the phosphorylation site has been digested away with the terminal residues. Considering also lysine-specific proteolysis (Fig. 1), which showed the phosphorylation site to be confined to the amino-terminal side of residue

238, the phosphorylation site must therefore be in the few residues nearest to the amino terminus. The trypsinisation work on *Spirodela* thylakoids showed that the digestion of D1 to T31 involves cleavage near the carboxy terminus [6]. The results reported here indicate that some amino-terminal residues have also been removed. This amino-terminal cleavage has recently been concluded from other studies involving D1 trypsinisation (Goloubinoff, P., personal communication).

The start of translation of the mRNA for D1 corresponds to the first 'AUG' codon of the open reading frame [7] giving the following amino-terminal sequences in pea and wheat:

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    1              5                  10
NH2-Met-Thr-Ala-Ile-Leu-Glu-Arg-Arg-Asp-Ser-
(pea)
NH2-Met-Thr-Ala-Ile-Leu-Glu-Arg-Arg-Glu-Ser-
(wheat)
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Following translation, some carboxy-terminal residues are removed, but the amino-terminal sequence is essentially retained in the mature D1 protein [6]. We presume that trypsinisation (to T31) removes the first seven or eight residues of the above amino-terminal sequence, including the phosphorylation site. Michel and Bennett [8] have shown that phosphorylation of Photosystem II polypeptides is essentially confined to threonine residues, and it is therefore most likely that the phosphorylated residue in D1 is threonine-2. In all plants, algae and cyanobacteria for which D1 has been sequenced, a threonine residue is conserved at this position, except in *Euglena gracilis* [9]. A threonine residue is also conserved at position 2 in the related D2 polypeptide of the Photosystem II reaction centre; this was recently suggested to be the phosphorylated residue in D2 [1]. Thus, both D1 and D2 are apparently phosphorylated at threonine-2 which is probably exposed on the stromal side of the membrane in both proteins. Bennett and coworkers have recently reached a similar conclusion based on analysis of phosphorylated peptides from Photosystem II; amino-terminal fragments of D1 and D2 were identified by mass spectrometry and found to contain *N*-acetyl-*O*-phosphothreonine [10]. The physiological role for this phosphorylation is as yet unclear, but could regulate the functional activity of photosystem II either by a direct effect on electron transport (e.g., ref. 11) or via changes in the organisation and assembly of the complex in the membrane.

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