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# Photosystem II reaction centers isolated from phosphorylated pea thylakoids carry phosphate on the D1 and D2 polypeptide subunits

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**The reaction centre of Photosystem II, composed of the D1/D2/cytochrome *b*-559 polypeptides, has been isolated from pea thylakoids after phosphorylation by a redox-activated kinase. It is shown that both the D1 and D2 polypeptides are phosphorylated using a lysine-specific protease which digests D2 and not D1. Proteolysis of phospho-D2 yields a 27 kDa non-phosphorylated fragment. By considering the digestion pattern in relation to amino acid sequence information it is concluded that D2 is phosphorylated near the amino terminus. The chromatographic procedures adopted for isolation of the reaction centre also resolved a highly phosphorylated fraction of the light-harvesting chlorophyll *a*/*b* complex.**

## Introduction

Several polypeptides of the thylakoid membrane have the property of being reversibly phosphorylated [1]. In this communication we describe the isolation of a phosphorylated chlorophyll protein complex believed to be the Photosystem II reaction centre [2–5] and localise the phosphorylation sites to the D1 and D2 polypeptides.

Recently a procedure has been developed to isolate a chlorophyll binding complex from spinach [2] which seems to be the PS II reaction centre [3–5]. In its most purified form the complex contains four or five chlorophyll *a* molecules, two pheophytin molecules, some β-carotene and about one haem of cytochrome *b*-559 [2]. It shows light induced absorption and EPR signals indicative of a PS II reaction centre [3–5]. A similar preparation has been obtained from pea [6]. Polypeptide analyses with specific probes [2,7] have shown that this complex consists of products derived from the chloroplast *psb*A, *psb*D, *psb*E and *psb*F genes. These genes code for the polypeptides D1 (herbicide binding protein, rapidly metabolised 32 kDa thylakoid protein), D2 and the α and β subunits of cytochrome *b*-559, respectively. In many different ways, this putative PS II reaction centre has functional and compositional characteristics similar to those of the reaction centre of purple bacteria [8–10].

The discovery that D1 and D2 form the reaction centre places particular importance on previous work which suggested that in higher plants the D2, and possibly the D1, polypeptides can be reversibly phosphorylated [1,11,12]. In this paper we have isolated and purified the PS II reaction centre from pea thylakoids which had been

Abbreviations: BBYs, Photosystem-II-enriched membranes (called after Berthold, Babcock and Yocum; Ref. 14); Chl, chlorophyll; D1, herbicide-binding protein (*psb* A gene product); D2, lysine-containing *psb* D gene product; LHC II, light-harvesting chlorophyll *a*/*b* complex; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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phosphorylated in the presence of [$\gamma^{32}$P]ATP. We demonstrate, by partial proteolysis using a lysine-specific protease, that in our preparations both the D1 and D2 polypeptides of the PS II reaction centre are phosphorylated and that the addition of one phosphate group (or more) has little or no effect on the apparent molecular weight of these proteins as judged by SDS-polyacrylamide electrophoresis or the ion-exchange properties of the complex.

## Materials and Method

Intact chloroplasts were isolated from 10–14-day-old pea plants (*Pisum sativum* var. Feltham first) [13] and osmotically shocked in 6 mM $MgCl_2$. The thylakoid suspension was then diluted with an equal volume of double strength medium to give a final composition of 0.33 M sorbitol, 10 mM Tricine, 3 mM $MgCl_2$ and 10 mM KOH (brought to pH 8.2 with HCl). The membranes were sedimented at $6000 \times g$ for 5 min and resuspended in the above medium with the $MgCl_2$ concentration increased to 10 mM. For protein phosphorylation, thylakoids were incubated at 0.25 mg Chl/ml in resuspension medium to which 0.5 mM ATP, 1 mg/ml sodium dithionite and (except where indicated) 10 mM NaF had been added. When radiolabelled membranes were required, 0.9–2.0 GBq [$\gamma^{32}$P]ATP per mg chlorophyll was also present. Incubations were carried out at 25 °C in the dark in a sealed flask for 45 min. Phosphorylation was terminated by centrifugation of the thylakoids ($6000 \times g$ for 5 min) at 4 °C followed by resuspension in Mes-NaOH (pH 6.0), 15 mM KCl, 10 mM $MgCl_2$ and 10 mM NaF. PS II-enriched membranes ('BBYs') were prepared from these thylakoids by the method of Berthold et al. [14], except that the chlorophyll-to-Triton X-100 concentration was lowered to a ratio of 1 : 16.5 and the second Triton treatment was omitted. The PS II-enriched fragments were stored at −80 °C in 10% glycerol, 20 mM Mes (pH 6.3), 15 mM NaCl and 5 mM $MgCl_2$.

For the isolation of PS II reaction centres, radiolabelled phosphorylated membranes (BBYs) were combined either with non-phosphorylated BBYs or with non-radioactive phosphorylated BBYs. The reaction centre isolation was carried out as described previously [6,7] by solubilization in 4% Triton X-100 followed by chromatography using DEAE-Fractogel (Merck-BDH). Fractions eluted from the column with a salt gradient were analysed for chlorophyll [15] and total $^{32}$P label. The latter was determined using a Nuclear Chicago end window gas flow counter. The polypeptide composition of individual fractions was analysed using 12–17% SDS-polyacrylamide gel electrophoresis as described previously [7] and radioactive bands were visualized by autoradiography.

## Results

Phosphorylation of thylakoids by the reductant-activated membrane-bound kinase was found to yield a number of phosphorylated polypeptides very similar to the pattern of labelling reported previously [1,12]. Grana-enriched membranes prepared from the phosphorylated thylakoids were further solubilized with Triton X-100 (see Materials and Methods) and applied to an anion-exchange column. Although extensive washing with buffer containing 20 mM NaCl and 0.2% Triton X-100 removes the bulk of the chlorophyll some of the labelled polypeptides remained bound. Fig. 1 shows the chlorophyll and radioactivity elution profiles obtained when the column was subjected to a salt gradient. Two major chlorophyll peaks were obtained. The first eluted with a peak at about 60 mM NaCl (fractions 34 to 44) while the second peaked at 130 mM NaCl (fractions 48 to 52). The radioactivity elution profile also contains two peaks. Interestingly, compared with the first chlorophyll peak, the first radioactive maximum eluted at a higher salt concentration (90 mM) including fractions 38 to 46. In contrast the second radioactive maximum coincided exactly with the latter chlorophyll peak.

Fig. 2A shows the Coomassie blue stained SDS-polyacrylamide gel electrophoresis profiles of some of the fractions eluted from the column together with their corresponding autoradiograms (Fig. 2B). In this experiment the radioactively labelled BBYs were combined with an approx. tenfold amount of similar non-labelled phosphorylated material. Hence the stained pattern represents predominantly non-phosphorylated protein whereas the autoradiogram indicates the

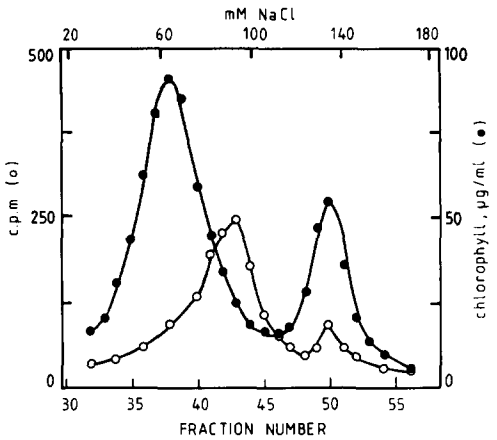


Fig. 1. Chromatography of Triton X-100 solubilized PS II-enriched membrane fragments (BBYs) obtained from pea chloroplast thylakoids. 0.5 mg of labelled phosphorylated material was combined with 8 mg of non-phosphorylated material before solubilization and application to an anion-exchange column. The column was washed with 50 ml of 50 mM Tris-Cl (pH 7.2), 0.2% Triton X-100 and 20 mM NaCl. 1 ml fractions eluted with a salt gradient were analysed for chlorophyll (●) and total $^{32}$P-label, cpm (○), as described in Materials and Methods.

phosphorylated bands. Track 5 represents the peak of the material that passed straight through the column, and the subsequent tracks (to fraction 30) the material eluted during the wash with 20 mM NaCl. Track 5 of the stained gel indicates that, although other polypeptides are present, the non-binding fraction is particularly rich in the light-harvesting complex (LHC II), polypeptides and the 33, 23, 17 kDa extrinsic polypeptides of the water-splitting complex. These polypeptides are not significantly labelled with $^{32}$P except for a trace of phospho-LHC II together with a labelled polypeptide of about 9 kDa molecular mass.

Tracks 32–57 represent the fractions eluted by the salt gradient. The first band of chlorophyll to elute from the column (fractions 34–44) is highly enriched in LHC II as judged by the SDS-polyacrylamide gel electrophoresis profiles in Fig. 2A. The autoradiogram (Fig. 2B) indicates that this LHC II is labelled with $^{32}$P, with the highest specific activity of labelling occurring in fractions eluted above 60 mM NaCl. Thus the polypeptide analysis and autoradiography support the data of Fig. 1 and suggest that LHC II eluted at 90 mM carries significantly higher levels of phosphate than does the major population eluting at 60 mM. The autoradiogram also shows that the only phosphorylated polypeptides, in the first chlorophyll peak of the elution profile, are those of LHC II.

The fractions in the second chlorophyll peak give an SDS-polyacrylamide gel electrophoresis pattern indicative of the PS II reaction centre, with a minor contamination from other polypeptide components. Antibody blotting shows that the bands at 30 and 32 kDa are due to D2 and D1 [7]. Immunoblotting also identifies the higher molecular-weight band at about 55 kDa as being composed of an aggregate of the D1 and D2 polypeptides [7]. The lower molecular-weight band at 9 kDa is due to the alpha subunit of cytochrome *b*-559 [2]. The autoradiogram of the fractions enriched in the PS II polypeptides shows radiolabelling coincident with both the D1 and D2 polypeptides and the 55 kDa aggregate. The autoradiogram also shows that the PS II reaction centre enriched fractions were contaminated with a low level of phospho-LHC II.

In order to confirm whether both D1 and D2 polypeptides were phosphorylated, a PS II reaction centre enriched fraction was treated with the proteolytic enzyme Lys-C, which specifically attacks lysine residues. Since the D1 polypeptide, unlike D2, does not contain lysine the enzyme should only attack D2 [7,12]. In Fig. 3 tracks a and b show the Coomassie blue staining of the PS II reaction centre fraction at two chlorophyll levels and the position of D1, D2 and their aggregated form, at 55 kDa, are indicated. Track d shows the effect of Lys-C treatment at a slightly lower chlorophyll loading and track c is the control incubated in the absence of the enzyme. The effect of proteolytic enzyme was to digest the contaminating LHC II polypeptides completely and also D2 as judged by the stained gel. In the latter case a D2 digestion product (confirmed by antibody labelling, see Refs. 7 and 16) appeared as a band at about 27 kDa. Lys-C digestion also slightly lowers the apparent molecular weight of the 55 kDa band corresponding to the D1/D2 aggregate. Autoradiograms of tracks c and d (i.e., tracks e and f, respectively) indicate that the radioactivity in the D1 and D2 region is considerably reduced after treatment with Lys-C. This is due to the loss of the phosphorylated D2 band. Radioactive

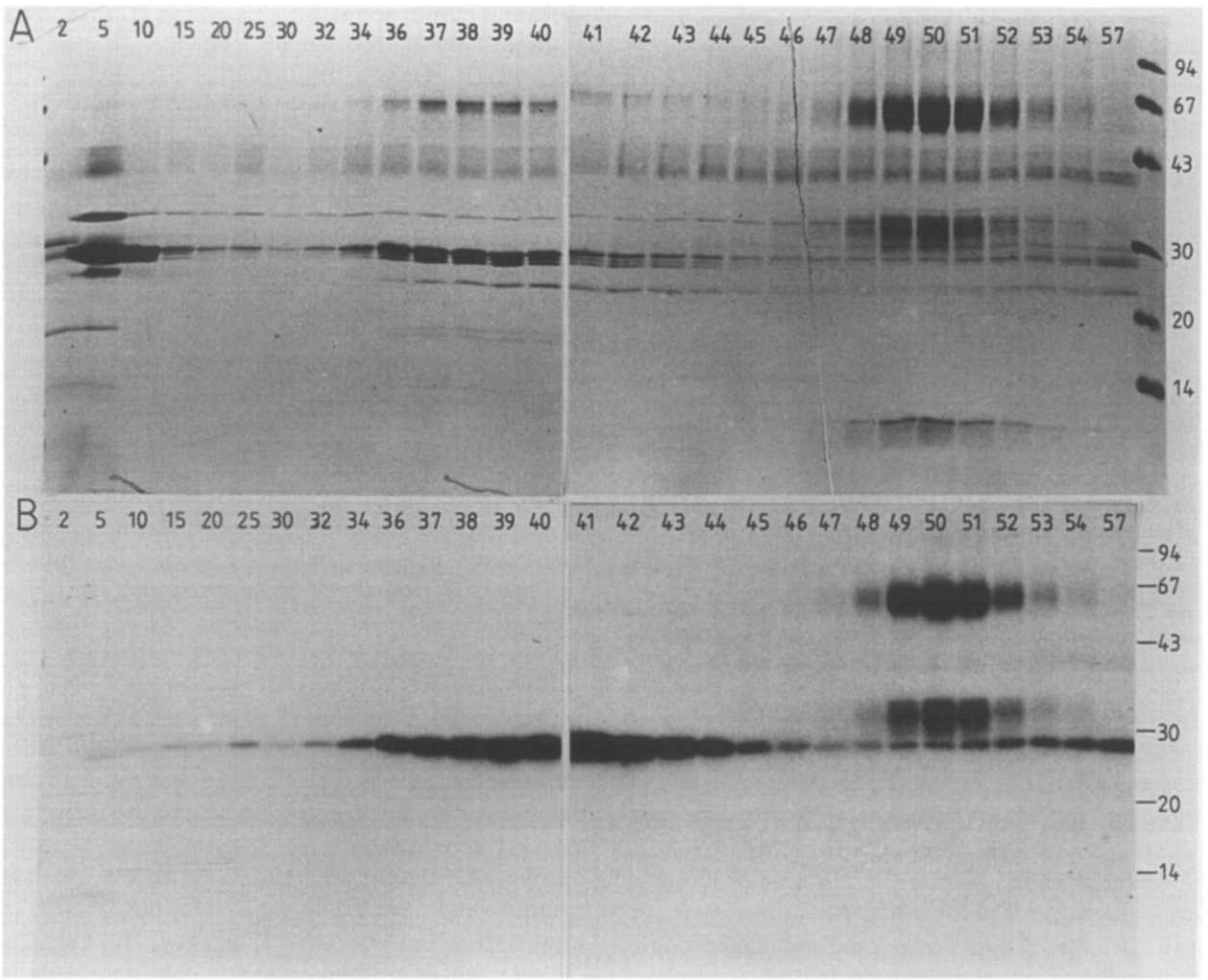


Fig. 2. SDS-polyacrylamide gel electrophoresis of various fractions obtained during the chromatographic separation of Triton X-100 solubilized PS II-enriched pea membranes (BBYs) as described in Fig. 1. (A) Coomassie blue stained gel; (B) Autoradiogram of the same gel lanes. Track 5 is typical of material passing straight through the column and subsequent tracks (to 30) are examples of material removed by washing the DEAE-Fractogel column with 20 mM NaCl. Tracks 32–57 are fractions sequentially eluted from the column by increasing the salt from 20 mM to 180 mM NaCl (see Fig. 1). Equal volumes of eluant (33 $\mu$l) were solubilized and loaded per track and approximate molecular masses in kDa are given at the side of the gels. Track numbers 0–30 correspond to the wash fractions (2 ml volume) and 32–57 to the salt gradient fractions (1 ml volume).

phosphate still remains on D1 and a more weakly labelled 28 kDa band. This band appears just above the position of the 27 kDa stained D2 digestion product.

We have found that the relative extent of phosphorylation of D1 and D2 varies considerably between experiments and, in an experiment where D2 labelling was particularly low, the 28 kDa phosphoprotein was visible both with and without Lys-C digestion (data not shown). We therefore conclude that this band is a phosphorylated form of the 28 kDa D1-related band previously demonstrated by $^{35}$S-methionine pulse-labelling and with D1-specific antibodies [7,16]. It appears that the cleavage of labelled D2 with Lys-C to 27 kDa totally removes the phosphorylated portion of the molecule.

The reaction centre preparation used in the experiment of Fig. 3 contains some contaminating material, mainly polypeptides of 40–43 kDa and

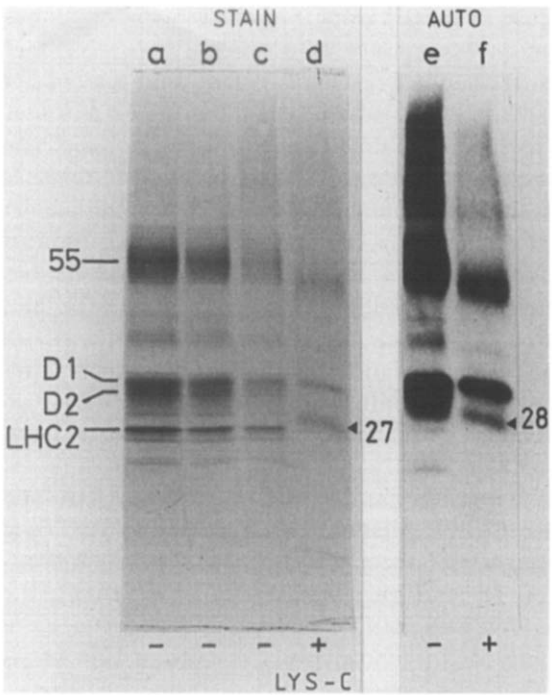


Fig. 3. Effect of the lysine-specific protease, Lys-C, on the polypeptide composition and $^{32}P$ distribution in a PS II reaction centre preparation. The reaction centres were prepared from a mixture of labelled (1 part) and unlabelled (3 parts) phosphorylated PS II enriched membranes (BBYs). Prior to Triton X-100 solubization at pH 7.2, the BBYs were washed with a high pH buffer (50 mM Tris, pH 9.0) in order to remove the extrinsic proteins associated with water splitting. Tracks (a)–(d) are Coomassie blue stained and derived from SDS-polyacrylamide gel electrophoresis. Tracks (e) and (f) are autoradiograms of (c) and (d), respectively. Chlorophyll loadings for (a) and (b) were 2.3 and 1.2 $\mu$g, respectively, while (c) and (d) were 0.9 $\mu$g chlorophyll. Track (d) shows the effect of treatment with 200 $\mu$g/ml Lys-C for 1 h at 37° C. The control sample, track (c), was treated in the same way as track (d), except that Lys-C was substituted by water. The stained bands before Lys-C treatment which correspond to the 55 kDa aggregate, D1, D2 and the major LHC2 polypeptide are marked. The stained 27 kDa band (lane d) and the labelled 28 kDa band (lane f) which are seen after Lys-C digestion are also marked.

of LHC II. The autoradiogram of the control lane (d) shows that the majority of the label is on the D1, D2 and 55 kDa bands. LHC II carried a very low level of labelling compared to the reaction centre polypeptides because in this particular experiment NaF was omitted during the phosphorylation of the initial starting material (cf. with data of Fig. 2). This exploits the fact that LHC II dephosphorylation is more rapid than that of the other phosphoproteins and hence the net phosphorylation level of LHC II is reduced relative to the other phosphoproteins [17]. Also to be noted is that the reaction centre preparation shown in Fig. 3 was isolated from radioactive phosphorylated membranes mixed with a larger quantity of non-radioactive phosphorylated membranes. Hence the stained gel contains a considerably higher proportions of phosphorylated proteins than in Fig. 2 where the bulk of the non-radioactive material was derived from membranes which were non-phosphorylated.

## Discussion

Several previous studies on intact thylakoids have show that more than one polypeptide of 30–35 kDa apparent molecular mass can be reversibly phosphorylated by a membrane-bound redox-activated kinase (e.g., Refs. 11 and 18). Using a PS II-enriched preparation from phosphorylated higher plant thylakoids, Michel and Bennett [19] observed two phosphopolypeptides which they suggested to be D1 and D2. However, using a PS II-core preparation Millner et al. [12] had previously concluded that only D2 is significantly phosphorylated. This discrepancy appears to be due to variation in the phosphorylation pattern, particularly with regard to the distribution of label between bands in the 30–35 kDa range. This could be partly due to the different methods used to activate the kinase (e.g., light, dithionite or NADPH and ferredoxin). However, we have found on repeating the experiments that even with the same activation method there was still some variability in the labelling.

The task of identifying the phosphorylated bands in thylakoids and even PS II-enriched grana preparations is complicated by the presence of several polypeptides with apparent molecular mass in the 30–35 kDa range. Millner et al. [12] attempted to overcome this by using a simpler PS-II-core preparation and testing the sensitivity of phosphorylated bands to the lysine-specific protease, Lys-C, to distinguish between D1 and D2. We have now repeated this exercise with a pre-

paration containing still fewer polypeptide constituents and show that PS II reaction centres, isolated from pea thylakoids which had been phosphorylated by the redox activated kinase are phosphorylated on both the D1 and D2 polypeptide subunits.

Phosphorylation makes no detectable difference to the mobilities of the polypeptides in SDS-polyacrylamide gel electrophoresis (see discussion of Fig. 2 in Results section). This result is at variance with the finding of Delepelaire [20] that phosphorylated D2 from the green alga, *Chlamydomonas reinhardtii*, migrates slightly more slowly during electrophoresis than non-phosphorylated D2. The phosphorylation also made no difference to the elution of the PS II reaction centre complex from the ion-exchange column (see discussion of Fig. 1 in Results section). Phospho-LHC II on the other hand appears to carry sufficient extra negative charge to shift its elution to a higher salt concentration. It should be noted, however, that there appear to be several populations of phospho- and non-phospho-LHC II which elute at different salt concentrations, a conclusion also reached by W. Kuehlbrandt (personal communication).

Treatment of the phosphorylated reaction centres with lysine-specific protease confirms our contention that both D1 and D2 carry phosphate groups. The phospho-D1 band is not digested by Lys-C which is in line with the known absence of lysine residues [21] and neither is the phospho-28 kDa polypeptide (modified D1) digested. On the other hand, the D2 polypeptide contains several lysine residues [22] and Lys-C digestion of phospho-D2 removes all of the label to leave only a non-phosphorylated 27 kDa fragment detectable by staining or antibody labelling. The phosphorylation site of D2 must therefore be on a proteolytic fragment of low molecular weight. The conclusion that both D1 and D2 are phosphorylated explains the occurrence of radiolabel in the 55 kDa band. The action of Lys-C is to decrease the size of the 55 kDa band by a few kDa as would be expected if it is composed of heterodimers of D1 and D2. Coupled with this lowering of molecular weight of the heterodimer band is a net decrease in radioactivity, presumably due to the loss of the phosphorylated fragment of D2. The D2 polypeptide of pea, as judged from the gene sequence [22], has four lysine residues at positions 7, 10, 265 and 318. We can be reasonably certain that the 27 kDa Lys-C digestion product contains at least residues 11 to 265. Since this fragment carries no label, the phosphorylation site(s) must be further towards one or both terminals. If we also assume that the site is exposed to the stroma, the organisation of D2 suggested in Refs. 9 and 10 would further restrict the possible site to residues 1–10 and 266–270. Michel and Bennett [19] have shown that phosphorylation of PS II polypeptides is restricted to threonine residues and pea D2 has two threonine residues in the relevant regions at positions 2 and 9 [22]. However, threonine 9 is absent in *Chlamydomonas* [23] and wheat (Dyer, T.A. and Barker, R.F., personal communication), but D2 is nevertheless phosphorylated in these organisms (Ref. 20, and our unpublished observation). We therefore suggest that threonine 2 is a strong contender for the phosphorylation site of D2. As far as D1 is concerned, it also has a widely conserved threonine residue at position 2 [21,24] and this may be its site of phosphorylation. In the light of these conclusions it is interesting to note that the LHC II apoprotein and the '9 kDa' phosphoprotein (see Ref. 19) are both phosphorylated on threonines nearest to the amino terminus.

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