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Localization and identification of phosphoproteins within the Photosystem II core of higher-plant thylakoid membranes

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Photosystem II core particles were isolated, using the method of Gounaris and Barber (Gounaris, K. and Barber, J. (1985) FEBS Lett. 188, 68–72) from [^{32}P]phosphate- and [^{35}S]methionine-labelled thylakoids of pea (*Pisum sativum*) chloroplasts. SDS-polyacrylamide gel analysis of the PS II core particles showed diffuse Coomassie blue stained polypeptide bands centred at molecular mass of 43, 40, 33, 30 and 10 kDa. Subsequent fluorography of the SDS-polyacrylamide gels revealed ^{32}P-containing bands at 55, 43, 40, 33 and 30 kDa and a ^{35}S-containing band, representing the D1 (herbicide-binding) protein, at slightly higher than 33 kDa. The 30 kDa [^{32}P]polypeptide which was the major ^{32}P-labelled species was therefore assigned to be the D2 protein, a conclusion reinforced by its degradation by the lysine-specific endoprotease, Lys-C. The approx. 33 kDa D1 protein, however, did not appear to be phosphorylated, since the weaker ^{32}P-containing 33 kDa band was degraded by the lysine specific enzyme, whilst the [^{35}S]D1 band was unaffected. Estimation of cytochrome *b*-559 in different fractions of the sucrose density gradient used to prepare PS II core particles indicated that the cytochrome was confined to the PS II core fraction, whereas the 10 kDa ^{32}P-labelled component was present in other fractions. Moreover, the 10 kDa ^{32}P-labelled polypeptide was readily degraded by the lysine-specific endoprotease, a result not expected for the lysine free cytochrome *b*-559 10 kDa polypeptide. It is, therefore, also concluded that the 10 kDa [^{32}P]component cannot represent the cytochrome *b*-559 apoprotein.

Introduction

Since the discovery of thylakoid-bound kinase activity by Bennett [1] it has become well established that the major phosphorylated polypeptides within the thylakoid membrane are components of the light harvesting complex (LHC II) [2,3,5]. The phosphorylation and dephosphorylation of these polypeptides provides a molecular mechanism whereby the distribution of quanta to each photosystem can be regulated [4–7]. However, in addition to the phosphorylation of the approx. 23–27 kDa LHC II polypeptides, the thylakoid-bound kinase(s) also catalyse phosphate incorporation into several other polypeptides, with the major phosphoproteins having apparent molecular masses around 45, 35–32 and 10 kDa [2,3,5]. The identities of the non-LHC II phosphopolypeptides have not yet been unequivocally demonstrated,

Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; D1, herbicide-binding protein (psbA gene product); D2, lysine-containing psb D gene product; LHC II, light-harvesting complex; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; Chl, chlorophyll; Q_A, first stable electron acceptor of Photosystem II.

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although there were indications that they are associated with Photosystem II (PS II) [8]. Indeed, the use of neutral detergents such as Triton X-100 has permitted the preparation of membrane subfractions highly enriched in PS II [9,10] and allowed us, as reported in a previous paper [11], to show that the major ^{32}P-labelled polypeptides were present in the PS II · LHC II supramolecular complex. Recently, there has been increased interest in isolating more purified PS II preparations along the lines of those pioneered by Satoh [12]. These procedures, together with the use of neutral detergents, such as octylglucoside [13,14] or Triton X-100 [15], in conjunction with chromatographic [14] or centrifugation [13,15] steps, allow the removal of LHC II and other polypeptides from PS II-enriched membranes to produce PS II 'core' particles which possess just a small number of polypeptides. In the present work we isolate such a preparation from ^{32}P-labelled PS II-enriched membranes to localize the major thylakoid phosphoproteins further. We have also utilized a number of biochemical criteria such as sensitivity to a specific protease and [^{35}S]methionine labelling to establish identities for the ^{32}P-labelled polypeptides.

Materials and Methods

Materials. [^{35}S]methionine was purchased from Amersham, U.K. and [γ-^{32}P]ATP from New England Nuclear, U.S.A. Lys-C endoprotease was purchased from Boehringer. All other chemicals were of 'AnalaR' or comparable grade. *Spirulina maxima* ferredoxin was a gift from Dr. K. Rao, King's College, London.

Thylakoid preparation. Prior to thylakoid preparation according to Ref. 16 pea plants (*Pisum sativum* var. Feltham first) were kept in darkness for 16–18 h. Following preparation, the thylakoid membranes were resuspended in a medium consisting of 0.33 M sucrose/20 mM Tricine-NaOH (pH 8.0)/15 mM NaCl/5 mM $MgCl_2$ and, immediately prior to use, were osmotically shocked in 10 mM $MgCl_2$ for 90 s before being recovered by centrifugation (5000 × *g* for 5 min) and again resuspended in the above-mentioned sucrose-tricine medium.

Protein phosphorylation. Broken thylakoids equivalent to 2 mg Chl were incubated under nitrogen in 20 ml of a medium consisting of 0.33 M sucrose/15 mM NaCl/20 mM Tricine-NaOH (pH 8.0) 10 mM $MgCl_2$/10 mM NaF/2.5 μM *Spirulina maxima* ferredoxin/0.5 mM NADPH/0.5 mM cold ATP/6.6–13.2 GBq [γ-^{32}P]ATP for 30 min in the dark at 20°C. The ^{32}P-labelled membranes were then recovered by centrifugation at 10000 × *g* for 10min and resuspended to 2 mg Chl · ml^{-1} in 2 mM Mes-NaOH (pH 6.0)/15 mM NaCl/5 mM $MgCl_2$/10 mM NaF.

[^{35}S]methionine labelling. Following dark preincubation, detached pea leaves (2–3 g) were floated on 5 ml of carrier-free [^{35}S]methionine (13.2 GBq · ml^{-1}) in 0.4% (v/v) Tween 20 in a sealed Petri dish. The leaves were then illuminated at a flux density of 55 μE · m^{-2} · s^{-1} for 2.5–3 h at 20°C, after which they were rinsed with deionized water and utilized for the preparation of thylakoids as described above.

Preparation of PS II-enriched membranes and PS II core particles. [^{32}P]phosphate- or [^{35}S]-methionine-labelled thylakoids, resuspended in 2 mM Mes-NaOH (pH 6.0)/15 mM NaCl/5 mM $MgCl_2$, plus 10 mM NaF for ^{32}P-labelled membranes, were used for the preparation of PS II-enriched membranes as described in Ref. 9. The PS II-enriched membranes were subsequently used to prepare PS II reaction centre core particles as in Ref. 15. This procedure involves centrifugation of Triton X-100 solubilised material on a 0.1–1.0 M sucrose density gradient. The Photosystem II reaction centre core particles (fraction 4) formed a band at about 0.65 M sucrose. Other fractions were located at 0.5 M sucrose (fraction 3), 0.3–0.4 M sucrose (fraction 2) and 0.13 M sucrose (fraction 1), respectively, and were harvested and stored in liquid nitrogen until required. Fraction 2 was readily identifiable as LHC II.

Lys-C protease treatment of ^{32}P- and ^{35}S-labelled sucrose gradient fractions. Aliquots (50 μl) of the reaction centre Photosystem II core particle fraction, or other gradient fractions were brought to pH 8.0 and 8 mM $MgCl_2$ by the addition of 1 M Tris base and 0.1 M $MgCl_2$, respectively. An aqueous solution of the Lys-C endoprotease (1 mg · ml^{-1}) was then added to give a protease concentration of 100 μg · ml^{-1} and serial dilutions carried out to give 10 and 1 μg · ml^{-1} Lys-C. An

equivalent volume of water was added to the control and the samples incubated for 1 h at 20°C. The protease digestion was terminated by adding 40 μl of electrophoresis solubilization buffer (see below) and freezing at −20°C.

SDS-polyacrylamide gel electrophoresis. ^{32}P- or ^{35}S-labelled samples were incubated with an approximately equal volume of solubilization buffer (4% (w/v) SDS/2% (v/v) mercaptoethanol/50 mM Tris (pH 8.7)/10% (w/v) sucrose) for 30 min at room temperature prior to electrophoresis on 7–17% polyacrylamide gradient gels as in Ref. 11. Staining of gels with Coomassie blue R250 was according to Ref. 17. Autoradiography of dried gels was carried out at −70°C using Fuji NIH X-ray film and high-speed intensifying screens. Fluorography of gels was carried out according to Ref. 18.

Determination of cytochrome b-559 in gradient fractions. Aliquots (100 μl) of Photosystem II reaction centre core particles, or other gradient fractions, were diluted to 1 ml in 50 mM Mes-NaOH (pH 6.5)/0.1 M NaCl/0.1% (v/v) Triton X-100. Cytochrome *b*-559 was determined from the dithionite-ferricyanide difference spectrum according to Ref. 19 using a Perkin-Elmer-557 double-beam spectrophotometer.

Results

Fig. 1 shows a typical Coomassie-blue stained gel of [^{32}P]phosphate- and [^{35}S]methionine-labelled pea thylakoids (lanes 2 and 3). Also shown, are PS II-enriched membranes (lanes 4 and 5) and sucrose density-gradient fractions labelled either with [^{32}P] (lanes 7–10) or [^{35}S] (lanes 11–13). Typically the PS II-enriched membranes (Fig. 1, lanes 4 and 5) show an enhancement of the intrinsic polypeptides having apparent molecular masses of about 43 and 40 kDa, 33–30 kDa (diffuse bands, D1 and D2, respectively), 27–24 kDa (LHC polypeptides) and 10 kDa (cytochrome *b*-559 apoprotein), and of the extrinsic polypeptides of 34, 23 and 17 kDa (see Ref. 10). With sucrose density-gradient fractions, the material apparent at the lowest density (fraction 1) consisted of several strongly Coomassie-stained polypeptides (Fig. 1, lanes 7 and 11). The fraction sedimenting to approx. 0.4 M sucrose (fraction 2) which contained most of the chlorophyll originating from the PS II membranes appeared to consist exclusively of a 27 kDa LHC II polypeptide (Fig. 1, lanes 8 and 12). The material appearing at the highest density, of around 0.65 M sucrose (fraction 4), represented Photosystem II reaction centre core particles. Typically, Coomassie blue staining after SDS-polyacrylamide gel electrophoresis of this material gives diffuse bands of around 43 and 40 kDa, 33–30 kDa (D1 and D2 polypeptides) and 10 kDa (*b*-559 apoprotein) (Fig. 1, lanes 10 and 13). However, variable amounts of polypeptides 34, 27 and 17 kDa were also seen. The latter components presumably represented contamination by extrinsic 'water splitting' complex polypeptides (34 and 17 kDa) and a LHC II polypeptide (27 kDa), respectively. It should be noted that material just above the Photosystem II reaction centre core fraction on the sucrose gradient was often observed (fraction 3) and gave the polypeptide pattern shown in Fig. 1, lane 9. However, fraction 3 was frequently present in insufficient amounts to permit fractionation as was the case for the ^{35}S-labelling experiment shown in Fig. 1. As expected, chlorophyll *a*/*b* ratios decreased from approx. 3.1 in thylakoid membranes to 2.1–2.4 in ^{32}P-labelled PS II membranes and 1.9–2.1 in ^{35}S-labelled PS II membranes. This difference in the chlorophyll *a*/*b* ratio between the two radiolabelled PS II-enriched membranes is probably due to changes in the degree of stacking induced by the extra negative charges resulting from the phosphorylation process [4]. The LHC II and Photosystem II reaction centre core fractions from both ^{32}P- and ^{35}S-labelled preparations possessed chlorophyll *a*/*b* ratios of 1.45 (±0.02) and 6.79 (±0.21), respectively.

In Fig. 2 a fluorograph of the SDS-polyacrylamide gel shown in Fig. 1, which contains both [^{32}P]phosphate- and [^{35}S]methionine-labelled material is presented. It is clearly evident that the major [^{32}P]phosphate-labelled polypeptides present in thylakoid membranes (Fig. 2, lane 2) were also present in the PS II-enriched membranes (Fig. 2, lane 4). However, when the [^{32}P]Photosystem II membranes were subsequently used to prepare Photosystem II reaction centre core particles, only polypeptides of 30 kDa and above, i.e., with

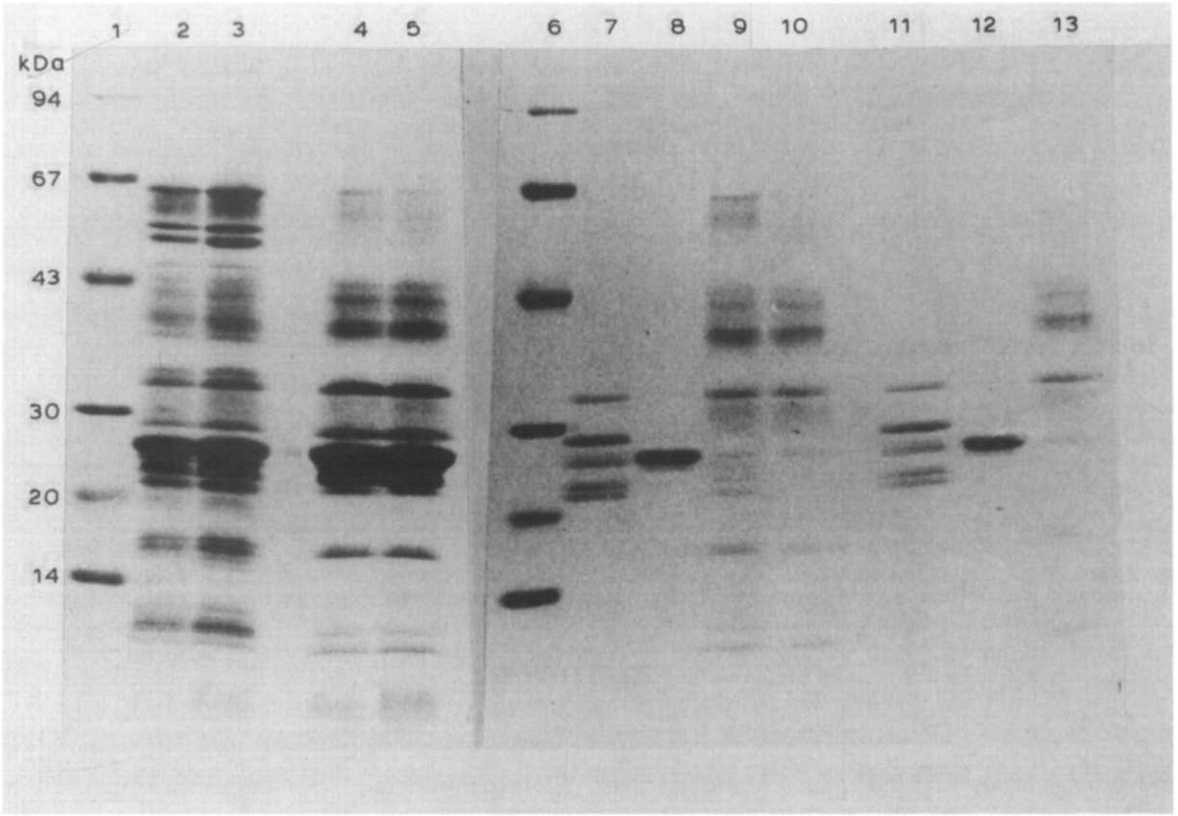

Fig. 1. SDS-polyacrylamide gel of thylakoids, PS II-enriched membranes and sucrose-density gradient fractions from the Photosystem II reaction centre core preparation procedure. Thylakoids and PS II preparations equivalent to 12 μg Chl and 25 μl of sucrose density gradient bands (except for fraction 2, where 5 μl was used) were loaded per lane after solubilizing with 25 μl of solubilization buffer (see Materials and Methods) at room temperature for 30 min. The gel was stained with Coomassie blue R250 and destained according to Ref. 17. Lanes 2 and 3, ^{32}P- and ^{35}S-labelled thylakoids, respectively; lanes 4 and 5, ^{32}P and ^{35}S-labelled PS II membranes; lanes 7–10, ^{32}P-labelled sucrose density gradient factions 1–4; lanes 11–13, ^{35}S-labelled sucrose density gradient fractions 1, 2 and 4, respectively. Lanes 1 and 6, molecular mass standards (Pharmacia).

molecular masses of 55, 43, 40, 33 and 30 kDa, were consistently observed (Fig. 2, lane 10). Small amounts of 27 kDa LHC II polypeptide, and a 10 kDa polypeptide were sometimes seen in this fraction. These latter ^{32}P-labelled components were, however, present at a much greater level in fractions sedimenting to a lower density (Fig. 2, lanes 7–9), although the 10 kDa component was never found in the LHC II fraction (Fig. 2, lane 8). With [^{35}S]methionine-labelled material, the major ^{35}S-labelled component which electrophoresed to approx. the same molecular mass as the 33 kDa phosphorylated component, was observed in thylakoids (Fig. 2, lane 3), PS II-enriched membranes (Fig. 2, lane 5) and reaction centre Photosystem II core particles (Fig. 2, lane 13). This component is known to be the D1 or 'herbicide-binding protein' which is rapidly turned over in the light [20,21] and thus becomes strongly ^{35}S-labelled under the conditions used here.

Since the apoprotein of cytochrome *b*-559 is known to possess a molecular mass of approx. 10 kDa [22–24] an assay for this cytochrome was carried out in the different sucrose density gradient fractions with the view of checking whether its distribution correlated with that of the ^{32}P-labelled 10 kDa component (Fig. 3). The dithionite-reducible cytochrome *b*-559 in these fractions was found to be almost exclusively confined to the Photosystem II reaction centre core-particle fraction (Fig.

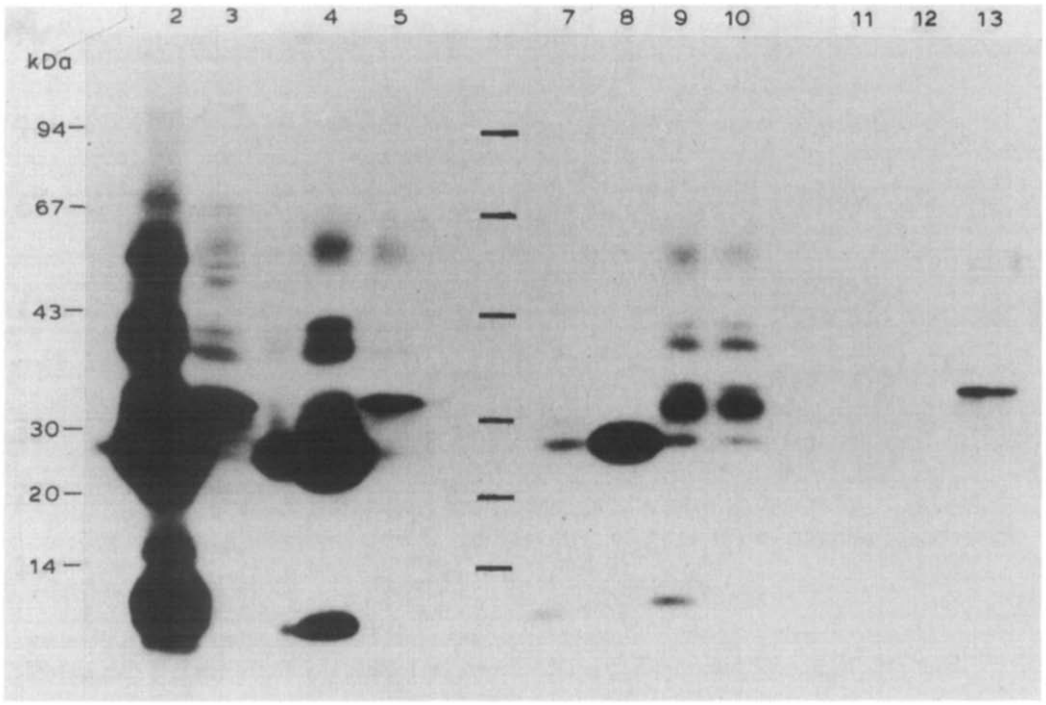

Fig. 2. Fluorograph of ^{32}P- and ^{35}S-labelled material shown in Fig. 1. After Coomassie blue staining (see legend to Fig. 1) gels were prepared for fluorography according to Ref. 18, and after drying, exposed at $-70\,^{\circ}C$. Lane numbers indicate same samples as in Fig. 1.

3, fraction 4), with little or no detectable cytochrome *b*-559 present in the other fractions (Fig. 3, fractions 1–3). In the Photosystem II reaction centre core fraction we detected on average 2 mol cytochrome *b*-559/65 ± 4 mol chlorophyll, although some samples possessed as little as 40 Chl/2 Cyt *b*-559, which was the ratio reported by Gounaris and Barber [15].

The chloroplast gene for the cytochrome *b*-559 10 kDa polypeptide (psb E gene) has been located and sequenced in spinach [25], wheat [26] and pea (Willey, D., personal communication). Although the gene product contains 83 amino acid residues, it completely lacks lysine. Also it is well known that the D1 herbicide-binding protein contains no lysine [27]. Therefore, these polypeptides should be resistant to attack by proteases which attack at lysine residues. In Fig. 4 the response of ^{32}P- and ^{35}S-labelled Photosystem II reaction centre core polypeptides to treatment with the lysine-specific endoprotease, Lys-C, is shown. As expected, the [^{35}S]methionine-labelled D1 polypeptide was insensitive to the endoprotease at up to 100 μg protease $\cdot$ ml^{-1} (Fig. 4, lanes 8–5). A ^{35}S-labelled polypeptide, at 55 kDa, and all of the ^{32}P-labelled components (Fig. 4, lanes 4–1), including the small amounts of 27 kDa LHC-II polypeptide and the 10 kDa polypeptide were degraded by this enzyme. The vulnerability of the 10 kDa phosphoprotein to the enzyme is shown more clearly in Fig. 5, where the sensitivity to LysC endoprotease of ^{32}P-labelled polypeptides in sucrose density gradient fractions 1 and 3, was tested. Here the LHC polypeptide and the 10 kDa component were clearly observed to be degraded by low concentrations (1 and 10 μg ml^{-1}, respectively) of the enzyme.

Discussion

The above results clearly indicate that the majority of the proteins which are rapidly phosphorylated under reducing conditions are associated with membranes enriched in PS II and LHC

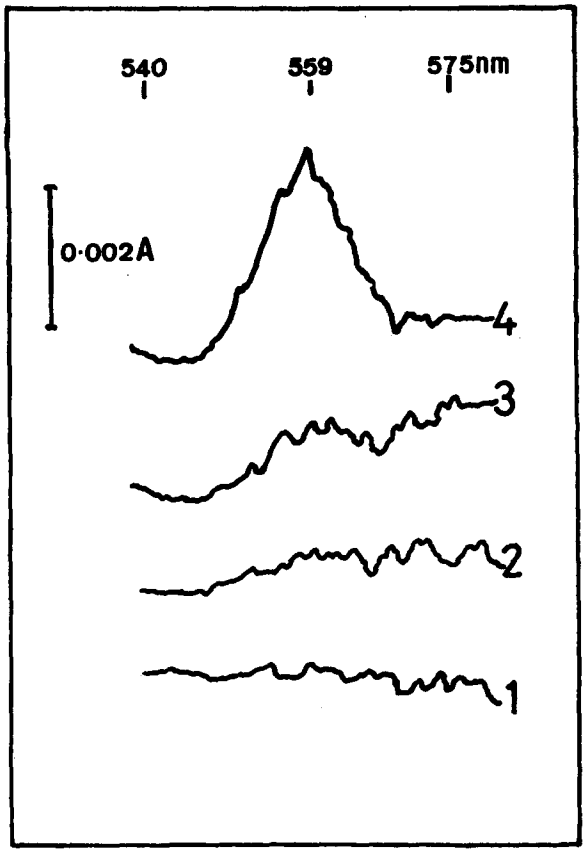

Fig. 3. Cytochrome *b*-559 in sucrose-density gradient fractions of a ^{32}P-labelled Photosystem II reaction centre core preparation preparation procedure. 100 μl aliquots of gradient fractions 1–4 were diluted to 500 μl with 0.1 M NaCl/0.1% (v/v) Triton X-100/50 mM Mes-NaOH (pH 6.5). Difference spectra of reduced (dithionite)-minus-oxidized (1 mM $K^3Fe(CN)^6$) samples were recorded using a Perkin-Elmer 557 double-beam spectrophotometer with a reference wavelength of 540 nm.

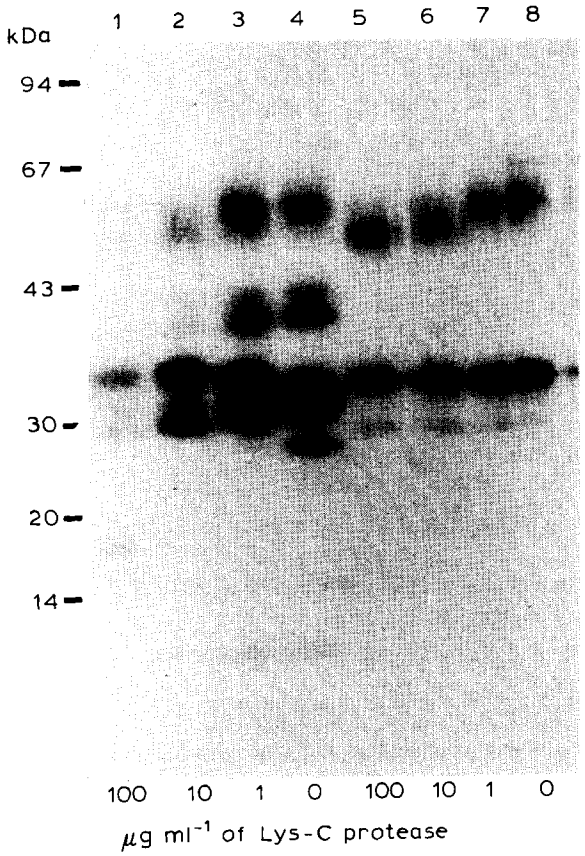

Fig. 4. Action of the lysine-specific protease, Lys-C, on Photosystem II reaction centre core particles labelled with either [^{32}P] or [^{32}S]. Aliquots, 50 μl, of Photosystem II reaction centre core particles were incubated for 1 h at pH 8.0 in the presence of the indicated concentrations of Lys-C prior to electrophoresis, staining and fluorography of the dried gels as described in Materials and Methods. Lanes 1–4, ^{32}P-labelled Photosystem II reaction centre core particles; lanes 5–8, [^{35}S]methionine-labelled reaction centre Photosystem II core particles. Molecular mass markers were as in Fig. 2.

II derived from the appressed granal regions. Further fractionation of these membranes has shown that several of the phosphoproteins are localized in the PS II core complex. Those which are absent or considerably reduced are the phosphorylated 27 kDa polypeptide of LHC II and a 10 kDa polypeptide. We have concluded that the 10 kDa phosphoprotein is not the apoprotein of cytochrome *b*-559, since its positioning in the sucrose density gradient, employed in the isolation of Photosystem II reaction centre core particles, did not correlate with the presence of the cytochrome. That the 10 kDa phosphoprotein is not the apoprotein of cytochrome *b*-559 was also argued by Widger et al. [22] based on separation of the phosphoprotein and cytochrome *b*-559. However, phosphorylation of a hydrophobic protein, such as cytochrome *b*-559, would most likely change its physical properties and could account for separation of phosphorylated and non-phosphorylated forms. Compelling evidence that the 10 kDa phosphoprotein is not cytochrome *b*-559 comes from our observation that the 10 kDa phosphoprotein was degraded by a lysine-specific endoprotease which is inconsistent with the known amino acid sequence of the cytochrome *b*-559 10 kDa apoprotein [23–26].

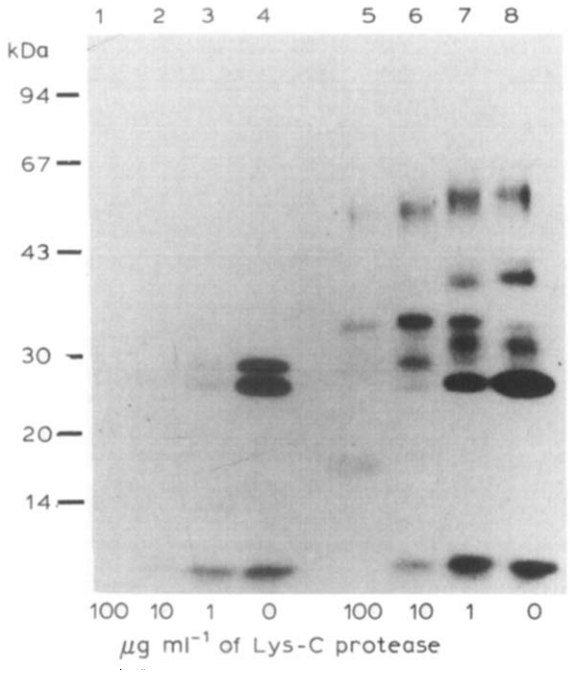

Fig. 5. Action of the lysine-specific protease, Lys-C, on ^{32}P-labelled sucrose density gradient fractions 1 and 3 (see Fig. 2, lanes 7 and 9). 50 μl aliquots of the gradient fractions were incubated for 1 h at pH 8.0 in the presence of the indicated Lys-C concentrations prior to electrophoresis, staining and autoradiography of the dried gels as described in Materials and Methods. Lanes 1–4, ^{32}P-labelled fraction 1; lanes 5–8, ^{32}P-labelled fraction 3. Molecular mass markers were as in Fig. 2.

Comparison of the [^{35}S]- and [^{32}P]fluorograms of gels derived from SDS-polyacrylamide gel electrophoresis of Photosystem II reaction centre core particles indicate that the radiosulphur-labelled D1 protein had an apparent molecular mass of about 33 kDa. In contrast, the strongest ^{32}P-labelled band in this molecular mass region was at 30 kDa, although a weaker radioactive band could be detected just above it. These findings suggest that the major phosphoprotein in the 30–33 kDa region is not the rapidly synthesised D1 herbicide binding protein. Further support for this contention also comes from the lysine-specific protease treatment. The data clearly shows that, unlike the 30 kDa phosphoprotein, the ^{35}S-labelled D1 polypeptide is not degraded by the enzyme. It therefore seems highly likely that the major phosphoprotein at 30 kDa is the lysine containing D2 protein [28–32]. A similar conclusion has been reached by Delepelaire with his studies on *Chlamydomonas reinhardtii* [33]. Although the weaker ^{32}P-labelled band at about 33 kDa could be the D1 protein its sensitivity to Lys-C endoprotease treatment suggests that it is not. It is possible that this weakly labelled component represents a partially dephospho-form of the D2 protein resulting from a dynamic equilibrium between the phospho- and dephospho-forms of this protein as suggested by Delepelaire [33]. It should be mentioned that the [^{32}P] activity which persisted just above 33 kDa after treamtent with 100 $\mu g \cdot ml^{-1}$ of protease is almost certainly the last remains of a degradation product of higher molecular-weight proteins (e.g., 43 and 40 kDa) more clearly seen with lower concentrations of the enzyme. Presumably these polypeptides are the products of the psb B and psb C genes which are known from sequencing to contain lysine residues [31,32,34].

The reason for the rapid phosphorylation of the PS II polypeptides under reducing conditions, corresponding to excess PS II light, is unknown. Particularly intriguing is the suggested phosphorylation of the D2 which, together with the D1 polypeptide, is likely to form the reaction centre of PS II by analogy with the L and M subunits of the bacterial system [35,36]. Certainly evidence exists which indicates that the protein phosphorylation process modifies the local-surface charge density at PS II and alters the rate of electron transfer in the reaction centre [37–39]. This modification of PS II activity could play a role in regulating energy dissipation under high light intensities as suggested by Horton and Lee [39]. Another important aspect of the work presented above is that we have demonstrated that the 10 kDa phosphoprotein can be separated relatively easily from the PS II core complex by using mild detergent treatment. Although this 10 kDa component is a major phosphoprotein and cannot be the apoprotein of cytochrome *b*-559, details of its precise origin and functions have as yet to be determined.

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