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Localization of a 64-kDa phosphoprotein in the lumen between the outer and inner envelopes of pea chloroplasts

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The identification and localization of a marker protein for the intermembrane space between the outer and inner chloroplast envelopes is described. This 64-kDa protein is very rapidly labeled by [γ-^{32}P]ATP at very low (30 nM) ATP concentrations and the phosphoryl group exhibits a high turnover rate. It was possible to establish the presence of the 64-kDa protein in this plastid compartment by using different chloroplast envelope separation and isolation techniques. In addition comparison of labeling kinetics by intact and hypotonically lysed pea chloroplasts support the localization of the 64-kDa protein in the intermembrane space. The 64-kDa protein was present and could be labeled in mixed envelope membranes isolated from hypotonically lysed plastids. Mixed envelope membranes incorporated high amounts of ^{32}P from [γ-^{32}P]ATP into the 64-kDa protein, whereas separated outer and inner envelope membranes did not show significant phosphorylation of this protein. Water/Triton X-114 phase partitioning demonstrated that the 64-kDa protein is a hydrophilic polypeptide. These findings suggest that the 64-kDa protein is a soluble protein trapped in the space between the inner and outer envelope membranes. After sonication of mixed envelope membranes, the 64-kDa protein was no longer present in the membrane fraction, but could be found in the supernatant after a 110000 × *g* centrifugation.

Chloroplasts are very complex cellular structures. While mitochondria have four different compartments (i.e. the outer membrane, the intermembrane space, the inner membrane and the matrix), chloroplasts have six compartments (i.e. the outer envelope membrane, the intermembrane space, the inner envelope membrane, the stroma, the thylakoid membranes and the thylakoid lumen). To date marker proteins have been identified for each chloroplast compartment, except for the intermembrane space between the outer and inner plastid envelope membranes.

In electron microscopic studies the interenvelope space can be seen as an electron-translucent space with an average width of 6 nm [1]. It is accessible from the cytoplasm to low-molecular-mass substances, which diffuse through large pores [2]. The inner envelope does not contain such pores and is impermeable to most low-molecular-mass molecules [3]. Except at some points where the outer and inner membranes form electron-dense areas, which are interpreted as contact sites [1, 4] there are no continuities observed between the outer and inner envelopes. To date we have no knowledge of the function of the intermembrane space, but from the observations described above it is likely that proteins in this compartment are involved in conveying the biochemical status and needs of the organelle to the cell and vice versa. Such signals have been postulated to occur, e.g. to regulate nuclear gene transcription [5].

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Abbreviation. LHCP, light-harvesting chlorophyll protein.

Enzymes. Phospholipase C (EC 3.1.4.3); lipase (EC 3.1.1.3); Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39).

Different methods are described in the literature to isolate chloroplast envelope membranes from green leaves [6 – 8]. If the separation of inner and outer envelopes is desired, chloroplasts are incubated in a hypertonic sucrose solution, which draws water from the stromal compartment and so widens the gap between the two membranes [1, 4] (see also Fig. 1). These hypertonically treated chloroplasts are then lysed by mechanical force (freeze/thaw cycle [9], Dounce homogenizer [7] or a Yeda press [10]) and membranes are separated by sucrose density gradient centrifugation. Soluble, hydrophilic proteins of the intermembrane space are most likely lost during this isolation procedure. However, when chloroplasts are swollen and lysed by resuspension in hypotonic buffer treatment, outer and inner envelope membranes can fuse at the breakage points [8] and retain at least some of the soluble protein material. We have used these approaches to identify such soluble intermembrane proteins and now report on a likely candidate, a 64-kDa phosphoprotein.

MATERIALS AND METHODS

Pea plants (*Pisum sativum* L., var. Golf) were grown in the greenhouse for two weeks.

[γ-^{32}P]ATP, 110 TBq/mmol was from Amersham-Buchler (Braunschweig). All other chemicals were of reagent grade and used without further purification.

Isolation of intact, purified chloroplasts and chloroplast subfractions

Chloroplasts were isolated from leaves of 14-day-old pea plants in low-ionic-strength buffer [11] and further purified

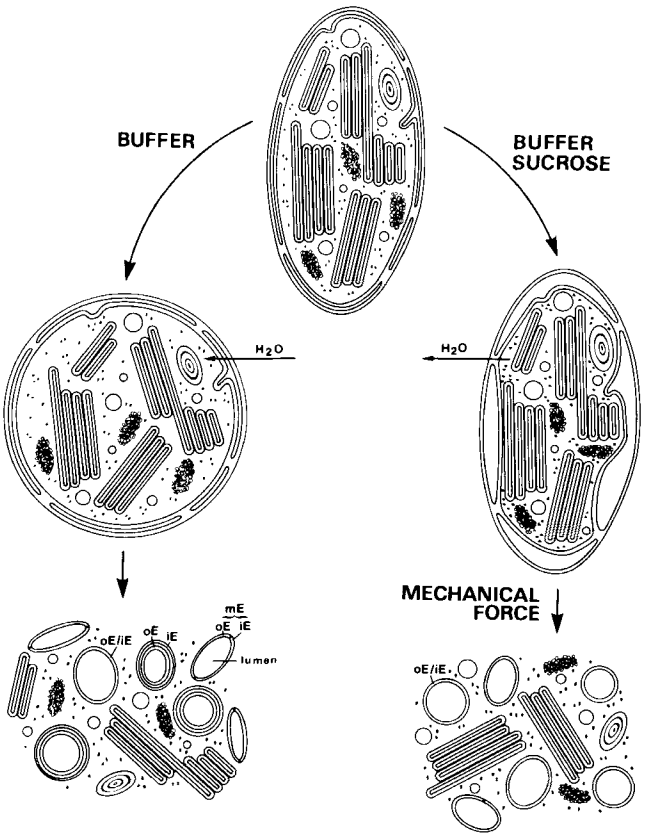

Fig. 1. *Differences in the chloroplast envelope membrane population after hypotonic or hypertonic treatment and lysis.* The scheme comprises data from [1, 4, 6, 8]. The different envelope membrane fractions are not represented in the correct relative amounts

on silica sol step gradients as in [12]. Chloroplast subfractions were isolated from intact, purified chloroplasts. A mixed envelope membrane fraction, in which vesicles were composed of material derived from both the inner and outer envelopes, was obtained by hypotonic lysis of the chloroplasts in 10 mM Tricine/KOH (pH 7.9) [3] and purification of the envelopes by a sucrose density gradient (3 h, $110000 \times g$) [3, 9]. Separation of inner and outer envelope membranes was achieved by hypertonic treatment of intact chloroplasts in 0.6 M sucrose. Shrunken chloroplasts were then ruptured by a Dounce homogenizer [7] and inner and outer envelope membranes separated by sucrose density gradient centrifugation (3 h, $250000 \times g$) [7]. Soluble chloroplast proteins were isolated from hypotonically ruptured chloroplasts and subjected to a high-speed centrifugation ($110000 \times g$, 1 h) prior to storage or use. Thylakoid membranes were recovered from the sucrose density gradients described above to isolate mixed envelope membranes. The sucrose concentration of this fraction was diluted to 0.5 M sucrose by 10 mM Tricine, pH 7.9 and thylakoids were pelleted for 3 min at $3000 \times g$. The pellet was resuspended and brought to a chlorophyll concentration of 0.1 – 0.2 mg chlorophyll ml^{-1}. Thylakoids were recovered by centrifugation (1 min, $3000 \times g$). The membranes were again resuspended and washed three more times at the same chlorophyll concentration and recovered by centrifugation in an Eppendorf microfuge for 10 s. Such vigorously washed thylakoid membrane fractions do not contain immunologically detectable levels of envelope contaminations (Soll and Eichacker, unpublished observation).

Protein phosphorylation

Protein phosphorylation was measured in the presence of 4 mM $MgCl_2$, 25 mM Tricine/KOH pH 7.9, 30 nM carrier-free [γ-^{32}P]ATP, chloroplast subfraction (5 – 10 µg protein) at 20 °C for 90 s in a final volume of 25 µl. The reactions were terminated by the addition of 30 µl twofold concentrated solubilization buffer [13]. Phosphorylation products were analyzed by SDS-PAGE [13] using a 10% or a 7.5 – 15% polyacrylamide separating gel. Gels were stained with Coomassie brilliant blue, destained in the presence of 20 mM KH_2PO_4, and the dried gel was autoradiographed overnight at −80 °C using an intensifying screen (Agfa-Gevaert MR-800, Agfa-Gevaert RP1). If necessary ^{32}P incorporation was quantified by excising the gel slices off the gel, rehydration with 0.5 ml H_2O and subsequent liquid scintillation counting.

Analysis of the 64-kDa phosphoprotein

Phosphoamino acid analysis of the 64-kDa phosphoprotein, labeled in the soluble chloroplast extract and the mixed envelope fraction, was done as in [14]. Envelope membranes and soluble proteins were labeled for 3 min under conditions described above. One half of the phosphorylation assay was analyzed by SDS-PAGE, the second half was precipitated by 200 µl 10% trichloroacetic acid. The pellet was washed three times with 5% trichloroacetic acid. Proteins were hydrolyzed in 6 M HCl at 110 °C for 2 h. Unlabeled phosphoamino acids (phosphotyrosine, phosphothreonine, phosphoserine, 20 µg each) were added to the reaction. The solvent was evaporated at 60 °C under a stream of N_2, resuspended in water, spotted onto a precoated Silica gel thin-layer plate (Merck, Kieselgel 60) and electrophoresed under cooling for 4 h at 1000 V using glacial acetic acid/formic acid/H_2O (78/25/897 by vol.) as running buffer. The phosphoamino acids were located by ninhydrin staining and radioactivity was detected by autoradiography as described above.

The 64-kDa soluble and envelope phosphoproteins were compared by partial proteolytic mapping [15] using V8 protease. Phosphorylated proteins were separated by SDS-PAGE, and dried gel slices were excised and reswollen in 125 mM Tris/HCl, pH 6.8, 1 mM EDTA, 0.1% (mass/vol.) SDS (buffer A) for 1 h. V8 protease (5 µl) was introduced into the wells of a second SDS/polyacrylamide gel (15% acrylamide), as a solution containing 0, 5, 25 or 500 µg enzyme/ml in buffer A supplemented with 10% (by vol.) glycerol, and allowed to electrophorese at 20 mA for 5 min.

The buffer was removed from the wells and replaced by 30 µl buffer A containing 20% glycerol and 0.001% bromophenol blue. After 1 min the buffer was removed and replaced by swollen gel fragments. The upper reservoir buffer was added and 5-µl aliquots of the V8 protease solutions were layered above the gel fragments. Electrophoresis was resumed, interrupted twice for 15 min, when the dye had travelled one-third and two-thirds of the way through the stacking gel, and continued until the dye had reached the end of the gel.

Sonication and Triton X-114 treatment

Soluble chloroplast proteins and envelope membranes were sonicated either before or after phosphorylation in a final volume of 100 µl on ice using a sonifier (Branson type 250) equipped with a microtip. Samples were sonicated five times for 2 s at 30 W. Following this treatment samples were centrifuged for 30 min at $110000 \times g$. In the case of the enve-

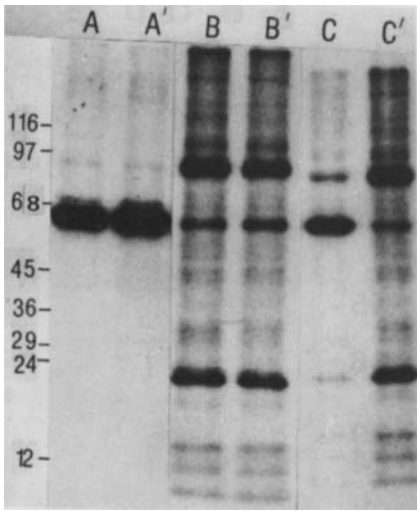

Fig. 2. *Phosphorylation patterns of mixed chloroplast envelope membranes with different ATP levels.* Products were analyzed by SDS-PAGE (7–15% polyacrylamide gradient) and autoradiography. The autoradiogram is shown. Lanes A,A′ were labeled with 33 nM [γ-^{32}P]ATP for 1 min and 2 min respectively; lanes B,B′ were labeled for 1 min and 2 min with 50 μM [γ-^{32}P]ATP; lane C: 1-min pulse with 33 nM [γ-^{32}P]ATP; C′: 1 min pulse as in C, followed by a 1-min chase with 50 μM ATP. Numbers indicate molecular mass markers in kDa

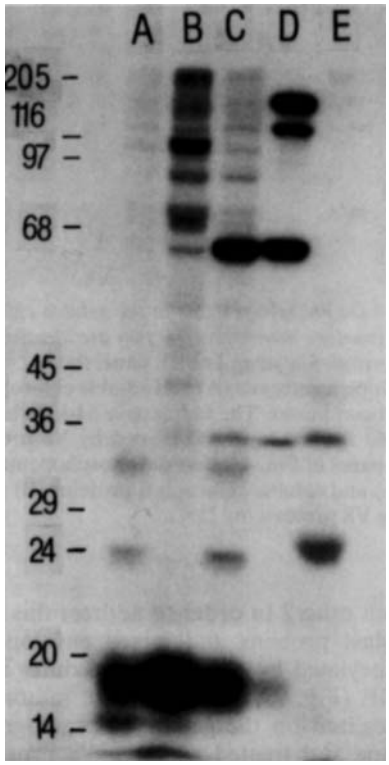

Fig. 3. *Different proteins are phosphorylated in isolated chloroplast subfractions.* Phosphorylation assays were done using identical protein contents (determined as in [17]). Chloroplast subfractions were phosphorylated for 90 s in the presence of 33 nM [γ-^{32}P]ATP. The phosphorylation patterns of inner envelope membrane (A), outer envelope membrane (B), mixed envelopes (C), soluble chloroplast proteins (D) and thylakoids (E) were compared. Fig. 3 shows the autoradiogram of SDS-PAGE (10% separating gel)

lope fraction, pellet and supernatant were separated and used for protein phosphorylation assays or further analyzed by SDS-PAGE if the phosphorylation reaction was done prior to sonication. The soluble protein extract was treated in the same manner, except that no pellet was visible after centrifugation and hence only the soluble phase was further analyzed.

Mixed envelope membranes were phosphorylated in 25-μl aliquots for 90 s. After this period the solution was brought to 100 μl and 5% Triton X-114 (by vol.) and left on ice for 30 min. The monophasic solution was then heated to 37 °C for a few minutes. This treatment results in a phase separation [16, 17]. The lower Triton X-114 phase and the upper aqueous phase were collected after a complete phase separation was obtained by centrifugation at $12000 \times g$ for 1 min. Aliquots of each phase were analyzed for phosphoproteins by SDS-PAGE. Part of the Triton X-114 phase was re-extracted with water under identical conditions and aliquots of each phase were analyzed by SDS-PAGE.

Miscellaneous

Protein was estimated by the procedure described in [18] using bovine serum albumin as a standard. Chlorophyll was determined as in [19].

RESULTS

When mixed envelope membranes were incubated with very different [γ-^{32}P]ATP concentrations, we observed dramatic differences in the phosphorylation patterns (Fig. 2). Using a very low [γ-^{32}P]ATP concentration (33 nM), a protein, which was phosphorylated to a significant extent, had an apparent molecular mass of 64 kDa on SDS-PAGE. Only when we used much higher ATP levels (50 μM) was the previously described [20] phosphorylation pattern observed. Under these conditions 86-kDa and 23-kDa proteins were the major phosphorylation products. It was possible to shift the phosphorylation pattern in a pulse-chase experiment. Mixed envelope membranes were first phosphorylated for 1 min using carrier-free [γ-^{32}P]ATP (33 nM). This resulted mainly in the phosphorylation of the 64-kDa protein (Fig. 2). Then the ATP level was raised by adding unlabeled ATP to a final concentration of 50 μM, and after a 1-min chase the phosphorylation pattern was identical to that described above using 50 μM ATP from the very start. The result indicates also that the 64-kDa protein has a very high phosphate turnover, since the amount of label in this protein was significantly decreased during the 1-min chase at 50 μM ATP (Fig. 2).

Initial attempts to localize the 64-kDa phosphoprotein using separated inner and outer envelope membranes showed that only very little of this phosphoprotein could be detected in these fractions compared with the labeling seen in mixed envelope preparations (Fig. 3). We extended our experiments to include other chloroplast subfractions by using thylakoids and soluble chloroplast proteins in the phosphorylation assay (Fig. 3). Surprisingly the soluble chloroplast protein fraction exhibited a major phosphorylated protein band with the same apparent molecular mass as that seen in the mixed envelope fraction, along with two other phosphoproteins with molecular masses of about 125 kDa and 180 kDa. The thylakoid membranes did not show any protein phosphorylation in the 64-kDa molecular mass region (Fig. 3).

Are the 64-kDa phosphoproteins in the soluble chloroplasts extract and the mixed envelope membrane fraction

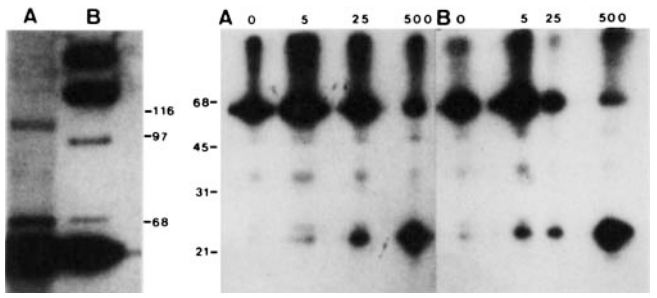

Fig. 4. *The 64-kDa phosphoproteins in the soluble chloroplast extract and the mixed envelope membrane fraction are identical as determined by proteolytic peptide mapping*. The left panel shows ^{32}P incorporation by mixed envelope membrane (A) and soluble chloroplast extract (B) under standard conditions. The radioactive 64-kDa bands of (A) and (B) were excised from the gel and treated by V8 protease digestion [15]. The right panel of Fig. 4 shows the phosphopeptides of envelope membranes (A) and soluble chloroplast proteins (B) generated using 0, 5, 25, 500 μg V8 protease/ml [15]

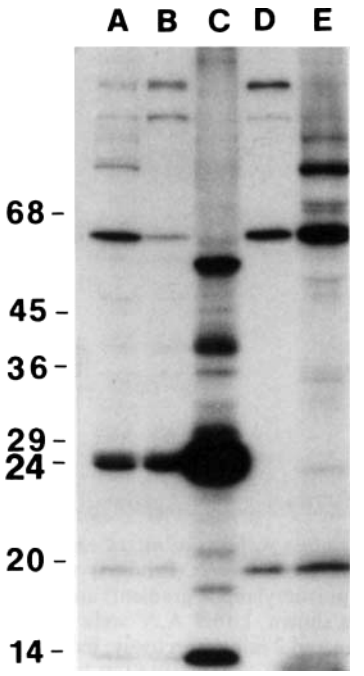

Fig. 5. *The phosphorylation capacities of intact chloroplasts and hypotonically lysed chloroplasts were compared to phosphorylation of chloroplast subfractions*. Chloroplasts were purified on silica sol gradients and lysed in the phosphorylation assay by omitting sorbitol from the resuspension buffer. Thylakoids were washed as described above. Total soluble protein was freed from any membrane particles by centrifugation at 110000 × *g* for 1 h. The autoradiogram shows protein phosphorylation of (A) intact chloroplasts (60 μg protein); (B) lysed chloroplasts (60 μg protein); (C) thylakoids (15 μg protein); (D) total soluble chloroplast protein (12 μg protein) and (E) mixed envelope membranes (10 μg protein). Phosphorylation was conducted for 90 s in the presence of 33 nM [γ-^{32}P]ATP

related to each other? In order to address this question, soluble chloroplast proteins and mixed envelope membranes were phosphorylated by standard procedures and subjected to SDS-PAGE (Fig. 4, left panel). The radioactive 64-kDa band was localized on the gel by autoradiography, excised from each lane and treated with the V8 protease during a second round of SDS-PAGE [15]. Separation of the proteolytic products revealed an identical phosphopeptide degradation pattern (Fig. 4, right panel). Phosphoserine was the only labeled amino acid detected when the two 64-kDa phosphoproteins were hydrolyzed and analyzed by high-voltage electrophoresis on silica gel thin-layer plates (data not shown). Thus, the 64-kDa proteins in the soluble extract and in the mixed envelope fraction are indeed identical.

From the data presented above two alternative conclusions could be drawn about the location of the 64-kDa phosphoprotein. It is either a stromal protein that adsorbs onto mixed envelopes but not onto separated inner and outer envelopes, or a soluble protein localized in the lumen between the outer and inner chloroplast envelopes. The second possibility is given greater credence if we consider the way in which envelope membranes are isolated. Mixed envelope membranes are isolated by hypotonic lysis of chloroplasts, and regions of the outer and inner membranes may fuse before the soluble intermembrane contents are completely lost [3, 8]. The soluble contents which escape during lysis will be recovered with the stromal proteins. When inner and outer membranes are separated by hypertonic treatment and mechanical disruption of the chloroplasts, entrapment of soluble interenvelope material is far less likely (see Fig. 1).

To distinguish between these possibilities, we compared the protein phosphorylation capacity of mixed envelope membranes, thylakoids, soluble chloroplast proteins, purified intact chloroplasts and hypotonically lysed chloroplasts in a phosphorylation experiment (Fig. 5). Intact chloroplasts showed major [^{32}P]protein labeling of a 24-kDa light-harvesting chlorophyll protein (LHCP), the 64-kDa protein and an 86-kDa protein identified previously as an outer envelope membrane protein [20]. Little label was present in the high-molecular-mass (125 kDa and 180 kDa) stromal phosphoproteins. When hypotonically lysed chloroplasts were used, the phosphorylation pattern changed in several ways: (a) label in the 64-kDa protein decreased significantly; (b) labeling of the 86-kDa envelope protein disappeared almost completely, and (c) labeling of the high-molecular-mass stromal proteins increased. Phosphorylation of the light-harvesting chlorophyll protein also increased slightly. Protein phosphorylation of washed thylakoid membranes showed strong ^{32}P incorporation into LHCP [21], the 10-kDa protein [21] and proteins of 38 kDa and 56 kDa; no overlap could be detected between the phosphorylation pattern of thylakoids and that of envelope membranes or total soluble chloroplast proteins. Soluble chloroplast proteins incorporate [γ-^{32}P]ATP into the two high-molecular-mass stromal proteins and also into polypeptides of 64 kDa and 19.5 kDa, while major phosphoproteins from mixed envelope membranes had apparent molecular masses on SDS-PAGE of 86 kDa, 64 kDa and 19.5 kDa. Mixed envelope membranes showed in no case significant protein phosphorylation of the high-molecular-mass stromal proteins (Figs 3–5, 7). The extent of ^{32}P incorporation differs for intact organelles and isolated subfractions. This can be seen in case of LHCP phosphorylation in chloroplasts and isolated thylakoids. While in Fig. 5, lanes A and B, the amount of thylakoids used is equivalent to about 30 μg thylakoid protein, phosphorylation is less than in lane C, which is equivalent to 15 μg isolated thylakoid protein. A similar observation can be made for the phosphorylation of the 64-kDa protein in intact chloroplasts (Fig. 5, lane A) and total soluble protein (Fig. 5, lane D). This could be due to a change in the specific activity of ATP, since intact and lysed chloroplasts probably still contain some residual endogenous ATP, while isolated subfractions do not.

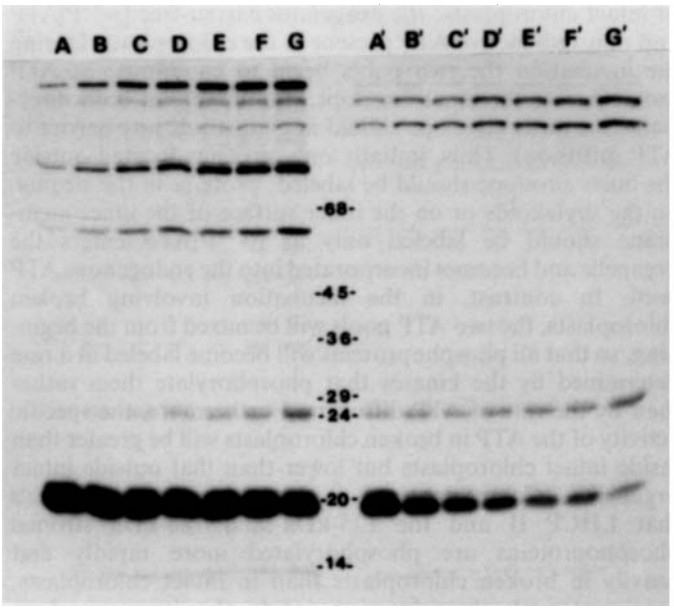

Fig. 6. *Time course of protein phosphorylation in (A–G) intact chloroplasts and (A′–G′) hypotonically lysed chloroplasts incubated in the presence of [γ-^{32}P]ATP.* Chloroplasts (equivalent to 300 µg protein) were incubated on ice for 10, 20, 30, 40, 50, 60, 90 s (A–G, A′–G′) in a final volume of 50 µl. Buffer and salt concentrations were as in Materials and Methods, except that incubations containing intact plastids contained 0.3 M sorbitol. At the indicated time intervals aliquots were removed and the reaction stopped by twofold solubilization buffer [19]

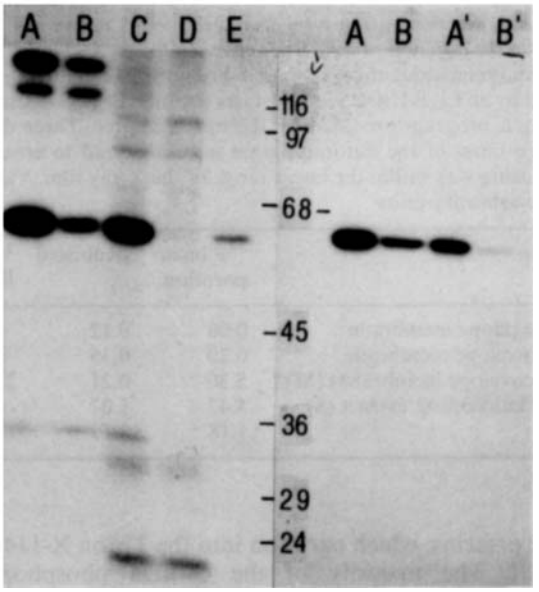

Fig. 7. *Effect of sonication and Triton X-114 treatment on the distribution of the 64-kDa phosphoprotein.* Protein phosphorylation was assayed before sonication (lanes A, C) or after sonication (lanes B, D, E) of soluble chloroplast proteins (A, B) and mixed envelope membranes (C, D, E). Envelope membranes were separated after sonication and prior to the phosphorylation assay into a pellet (D) and a supernatant (E) (110000 × *g*, 30 min). Equal amounts of each fraction (D, E) were used in the phosphorylation assay. All other conditions as in Materials and Methods. The right panel shows the aqueous (A) and Triton X-114 (B) phase after phase partitioning of a standard phosphorylation reaction. The Triton X-114 phase (B) was re-extracted once with water (A′, B′) and analyzed as above by SDS-PAGE and autoradiography. A′, second aqueous phase; B′, re-extracted Triton X-114 phase shown in B

These results establish that the 64-kDa protein resembles the 86-kDa outer envelope protein more than the 125-kDa and 180-kDa stromal proteins with respect to phosphorylation behavior and localization.

Confirmation is provided by the time-course experiment presented in Fig. 6. The rationale behind this approach is that carrier-free [γ-^{32}P]ATP should be incorporated into envelope polypeptides first and only later into internally localized proteins. Secondly, upon lysis of the chloroplasts, non-radioactive ATP present in the chloroplast should mix with exogenous [γ-^{32}P]ATP, lowering the specific radioactivity experienced by the envelope protein kinases but increasing the specific radioactivity of stromal proteins. In order to slow both the transport of [γ-^{32}P]ATP into the chloroplast and the labeling kinetics, phosphorylation experiments were carried out at 4 °C (compared with 20 °C in Fig. 5). This regime permitted us to observe differential labeling of polypeptides from intact and broken chloroplasts (Fig. 6). Intact, purified chloroplasts showed significant protein phosphorylation of the outer-envelope-membrane-bound 86-kDa protein, the 64-kDa protein and the 19.5-kDa protein, while LHCP and the 125-kDa and 180-kDa stromal phosphoproteins were labeled more slowly. In contrast, hypotonically lysed chloroplasts showed strong protein ^{32}P-labeling of the LHCP and the high-molecular-mass stromal proteins even at the earliest time points, while phosphorylation of the 86-kDa, 64-kDa and 19.5-kDa proteins was reduced dramatically. The nature of the 19.5-kDa protein and the reason for its heavy labeling at 4 °C are currently under investigation.

In order to characterize further the nature of the 64-kDa protein we sonicated a mixed envelope fraction and separated it into a membrane fraction and a supernatant fraction by centrifugation at 110000 × *g*. Both fractions were used in the standard protein phosphorylation assay. The results (Fig. 7) revealed that the 64-kDa protein was almost exclusively located in the supernatant while other envelope phosphoproteins where still membrane-bound. Stromal contaminants, however, were not released from the envelope membranes by sonication, as was judged from the distribution of the large subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) before and after sonication. About 8–10% of the large subunit appear in the 110000 × *g* supernatant, as determined by densitometry of Coomassie-brilliant-blue-stained SDS-PAGE, even without sonication, but none of the 64-kDa phosphoprotein appeared in the supernatant prior to sonication. The release of Rubisco was not significantly increased by the sonication regime we used. Sonication of envelopes resulted in a partial loss of protein kinase activity. This was true also when the soluble chloroplast extract was sonicated (Fig. 7). When mixed envelope membranes were sonicated after incubation with [γ-^{32}P]ATP the 64-kDa phosphoprotein was exclusively recovered in the supernatant (not shown). A treatment of mixed envelope membranes (2 mg/ml, pH 7.9) with phospholipase C (120 U/ml) and lipase (1500 U/ml) for 30 min at 20 °C resulted also in the release of the 64-kDa phosphoprotein into the 110000 × *g* supernatant. The 86-kDa phosphoprotein remained in the pellet fraction (not shown).

To examine whether the 64-kDa polypeptide was hydrophobic or hydrophilic a mixed envelope preparation was subjected to Triton X-114/water phase partitioning after phosphorylation. This technique separates hydrophilic polypeptides, which partition into the water phase, from hydro-

Table 1. *Comparison of ^{32}P incorporation in the 64-kDa protein shown in Fig. 3 in relation to the presence of the large subunit of Rubisco present in the different protein fractions used in this assay*
Autoradiograms and the Coomassie-brilliant-blue stained gel were scanned by an LKB Ultro Scan XL laser scanner. Peaks were integrated using a program provided by the manufacturer. Three different exposure times of the autoradiogram were analyzed to ensure that the exposure was within the linear range of the X-ray film. Values are given in arbitrary units

Fraction	^{32}P incorporation	[Rubisco]	^{32}P/Rubisco
Inner envelope membrane	0.06	0.12	0.5
Outer envelope membrane	0.20	0.35	0.57
Mixed envelope membranes (M)	5.30	0.21	25.2
Soluble chloroplast extract (S)	4.47	1.07	4.2
M/S	1.18	0.19	–

phobic proteins, which partition into the Triton X-114 phase [16, 17]. The majority of the 64-kDa phosphoprotein partitioned into the hydrophilic phase (Fig. 7, right panel). The ratio of label in the aqueous phase to the Triton phase was 2.7. When the Triton phase was extracted a second time with water, most of the residual label partitioned into the aqueous phase and ratio of label in the aqueous phase and the Triton phase was again 2.7 (Fig. 7, right panel). This ratio was determined after SDS-PAGE by excising the labeled bands off the gel and subjecting them to liquid scintillation counting.

DISCUSSION

The present study provides evidence that a 64-kDa phosphoprotein is most likely localized in the intermembrane space between the outer and inner envelopes of pea chloroplasts. The results show that the 64-kDa protein is a hydrophilic and soluble protein, which upon plastid lysis can be found in both the mixed envelope membranes and the soluble protein extract. There are two reasons for doubting that the 64-kDa protein in mixed envelope membranes can be explained by general stromal contamination. First, two other phosphoproteins (about 125 kDa and 180 kDa), which are clearly located in the stroma, are not found to contaminate envelope preparations (Fig. 4). Secondly, the specific labeling of the 64-kDa protein per unit of Rubisco (Table 1) is sixfold higher in mixed envelope preparations than in total soluble protein preparations. Of course, these two results could be explained by supposing that the 64-kDa protein has a specially high affinity for envelopes (either functionally *in vivo* or artefactually *in vitro*), but then it would be difficult to understand why the 64-kDa protein has little tendency to bind to separated inner and outer envelopes, while Rubisco binds avidly to these membranes (Table 1). It is much easier to explain these results by supposing that the 64-kDa protein resides in the space between the inner and outer envelopes and is retained during mixed envelope preparation under hypotonic conditions but lost during separation of the two envelopes under hypertonic conditions, or during sonication of mixed envelopes.

Further evidence in favour of the location of the 64-kDa protein between the envelopes is provided by our kinetic comparison of phosphorylation in intact and broken chloroplasts (Fig. 6). Two pools of ATP exist at the start of the incubation of intact chloroplasts: the exogenous carrier-free [γ-^{32}P]ATP and non-radioactive ATP present in the chloroplasts. During the incubation the two pools begin to equilibrate as ATP moves across the inner envelope, presumably in both directions (the outer envelope should not constitute any barrier to ATP diffusion). Thus, initially only proteins located outside the inner envelope should be labeled; proteins in the stroma, on the thylakoids or on the inner surface of the inner membrane should be labeled only as [γ-^{32}P]ATP enters the organelle and becomes incorporated into the endogenous ATP pool. In contrast, in the incubation involving broken chloroplasts, the two ATP pools will be mixed from the beginning, so that all phosphoproteins will become labeled at a rate determined by the kinases that phosphorylate them rather than by the rate of ATP diffusion. Furthermore, the specific activity of the ATP in broken chloroplasts will be greater than inside intact chloroplasts but lower than that outside intact organelles. Comparison of the two incubations reveals that LHCP II and the 125-kDa and 180-kDa stromal phosphoproteins are phosphorylated more rapidly and heavily in broken chloroplasts than in intact chloroplasts, consistent with their location inside the inner envelope (Fig. 6). The 86-kDa protein, by contrast, is phosphorylated more rapidly and heavily in intact chloroplasts, consistent with its location on the outer envelope [20]. The 64-kDa protein resembles the 86-kDa protein in being labeled more rapidly and heavily in intact chloroplasts, so it cannot be located in the stroma but could be located either at the outer surface of the inner envelope, on the outer envelope, or in the space between the envelopes. Since the 64-kDa protein does not appear in purified inner or outer envelopes, we conclude that it is located in the lumen between them.

Reports from different laboratories [24, 25] of a 67-kDa phosphoprotein in the stroma fraction (soluble protein fraction, by our definition) from spinach chloroplasts might, in fact, have identified the luminal phosphoprotein, which in spinach has a molecular mass of 67 kDa (Bennett and Soll, unpublished). Definite localization of the 64-kDa phosphoprotein has to await electron microscopic studies using immunogold labeling. This method allows the specific localization of proteins *in situ* when an antibody is available.

At present we can only speculate on the function of the 64-kDa phosphoprotein. It is not clear at the moment whether it is a subunit of a protein kinase undergoing autophosphorylation or whether it is a protein substrate with a very high phosphoryl-group turnover rate. Experiments designed to solve this problem are currently under way. Both chloroplast envelope membranes house phosphoproteins and protein kinases [20, 23]. The 64-kDa protein might be involved in signal transduction either between the two envelope membranes or between the inner envelope and the cytoplasm, which is connected to the intermembrane space by large pores [2]. Inner and outer envelope are in contact only at few sites [1], which are thought to be involved in post-translational protein uptake into the organelle [6, 26]. The possibility that the 64-kDa phosphoprotein is identical with the Rubisco-large-subunit-binding protein, which appears to be active in the assembly of the holoenzyme [27, 28], was excluded by Western blotting experiments (Bennett et al., unpublished results).

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REFERENCES

1. Carde, J. P., Joyard, J. & Douce, R. (1982) *Biol. Cell 44*, 315–324.
2. Flügge, U. I. & Benz, R. (1984) *FEBS Lett. 68*, 259–262.
3. Douce, R. & Joyard, J. (1979) *Adv. Bot. Res. 7*, 1–116.
4. Cline, K., Keegstra, K. & Staehlin, L. A. (1985) *Protoplasma 125*, 111–113.
5. Oelmüller, R., Levitan, J., Bergfeld, R., Rajaschar, V. K. & Mohr, H. (1986) *Planta (Berl.) 168*, 482–492.
6. Douce, R., Block, M. A., Dorne, A. J. & Joyard, J. (1984) *Subcell. Biochem. 10*, 1–84.
7. Keegstra, K. & Youssif, A. E. (1986) *Methods Enzymol. 118*, 316–325.
8. Douce, R. & Joyard, J. (1982) in *Methods in chloroplast molecular biology* (Edelmann, M., Hallick, R. B. & Chua, N.-H., eds) pp. 239–256, Elsevier, Amsterdam.
9. Cline, K., Andrews, J., Mersey, B., Newcomb, E. H. & Keegstra, K. (1981) *Proc. Natl Acad. Sci. USA 78*, 3595–3599.
10. Block, M. A., Dorne, A. J., Joyard, J. & Douce, R. (1983) *J. Biol. Chem. 253*, 13273–13280.
11. Nakatani, H. J. & Barber, J. (1977) *Biochim. Biophys. Acta 461*, 510–512.
12. Mourioux, G. & Douce, R. (1981) *Plant Physiol. 67*, 470–473.
13. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
14. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl Acad. Sci. USA 77*, 1311–1315.
15. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.
16. Ljungberg, U., Henrysson, T., Rochester, C. P., Åkerlund, H. E. & Andersson, B. (1986) *Biochim. Biophys. Acta 849*, 112–120.
17. Bourdier, C. (1981) *J. Biol. Chem. 256*, 1604–1607.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
19. Arnon, D. J. (1949) *Plant Physiol. 24*, 1–15.
20. Soll, J. (1985) *Planta (Berl.) 166*, 394–400.
21. Bennett, J. (1977) *Nature (Lond.) 269*, 344–346.
22. Werner-Washburne, M., Cline, K. & Keegstra, K. (1983) *Plant Physiol. 73*, 569–575.
23. Soll, J. & Buchanan, B. B. (1983) *J. Biol. Chem. 258*, 6686–6689.
24. Guitton, C. & Mache, R. (1987) *Eur. J. Biochem. 166*, 249–254.
25. Cortez, N., Lucero, H. A. & Vallejos, R. H. (1987) *Arch. Biochem. Biophys. 254*, 504–508.
26. Schleyer, M. & Neupert, W. (1985) *Cell 43*, 339–350.
27. Bloom, M. V., Milos, P. & Roy, H. (1983) *Proc. Natl Acad. Sci. USA 80*, 1013–1017.
28. Barraclaugh, R. & Ellis, R. J. (1980) *Biochim. Biophys. Acta 608*, 19–31.