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LABORATOIRE DE PHYSIOLOGIE VEGETALE — PROF. P.A. SIEGENTHALER

LES PROTEINES DE L'ENVELOPPE DU CHLOROPLASTE D'EPINARD : IDENTIFICATION ET CARACTERISATION DE L'ADENOSINE TRIPHOSPHATASE.

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Les protéines de l'enveloppe du chloroplaste d'Epinard: identification et caractérisation de l'adénosine triphosphatase

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RESUME

Les membranes d'enveloppe isolées à partir des chloroplastes d'Epinard contiennent 0.2% de protéines chloroplastiques totales. L'enveloppe est constituée d'une vingtaine de protéines dont les pI varient de 7.7 à 5. Ces protéines peuvent se décomposer en 60 polypeptides au moins, dont les poids moléculaires vont de 170 à 16 kD. Parmi ces polypeptides se trouvent les deux sous-unités de la RuBPCase (54 et 16 kD), et le translocateur de trioses-P/phosphate (29 kD).

L'enveloppe du chloroplaste d'Epinard renferme une activité d'ATPase. Elle se distingue des ATPases du thylacoïde, de la mitochondrie, de la membrane plasmique et de la vacuole. L'ATPase de l'enveloppe chloroplastique est activée par le $MgCL_2$, le $MnCl_2$, le $CaCl_2$, la calmoduline, le Triton X-100 et l'action de la phospholipase A_2. Son activité a un pH optimal de 7.8 et présente une énergie d'activation de 15 kcal/mole. Son activité spécifique est de l'ordre de 200 nmoles Pi/min/mg protéine en présence de concentrations équimolaires de $MgCl_2$ et d'ATP ($MgCl_2$ = ATP $\geq$ 4 mM). Elle est plus forte au printemps et en automne que pendant le reste de l'année. Le K_M pour $MgATP^{2-}$ est de 0.55 mM.

L'ATPase de l'enveloppe chloroplastique est insensible au NaN_3, au p-trifluorométhoxy-phénylhydrazone, à l'ouabaïne, au dicyclohexylcarbodiimide, à la phlorizine, à la quercétine, au diéthylstilbestrol et au molybdate. Par contre, elle est inhibée par l'ADP, l'oligomycine, le Na_3VO_4 et le $LaCl_3$. Son site actif contient des résidus de cystéine, de tyrosine et de tryptophane.

L'ATPase de l'enveloppe chloroplastique est probablement un tétramère de 260 kD dont les monomères ont un poids moléculaire de 65 kD et des points isoélectriques de 7.3 et 6.

L'ATPase n'est apparemment pas impliquée dans les échanges de H^+, K^+, Na^+ ou Ca^{2+}. Elle participerait au transport, à travers l'enveloppe chloroplastique, des protéines synthétisées par des ribosomes cytoplasmiques à la lumière.

LISTE DES TRAVAUX SCIENTIFIQUES

COMMUNICATIONS SCIENTIFIQUES :

Nguyen TD and Siegenthaler PA (1982). Proteins of spinach chloroplast envelopes. Experientia 38, 729.

Nguyen TD and Siegenthaler PA (1983). Isolation of a Mg^{2+}-ATPase from spinach chloroplast envelopes using a calmodulin affinity column. Experientia 39, 651.

Schwitzguébel JP, Nguyen TD and Siegenthaler PA (1983). Calcium, calmodulin and the oxidation of exogenous NADH by higher plants mitochondria. Experientia 40, 614.

PUBLICATIONS SCIENTIFIQUES :

Siegenthaler PA and Nguyen TD (1983). Proteins and polypeptides of envelope membranes from spinach chloroplasts. I. Isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis separations. Biochim. Biophys. Acta 722, 226-33.

Nguyen TD and Siegenthaler PA (1983). Proteins and polypeptides of envelope membranes from spinach chloroplasts. Properties of a membrane-bound ATPase. FEBS Lett. 164, 67-70.

Nguyen TD and Siegenthaler PA (1984). A (Mg^{2+}- Ca^{2+}) stimulated ATPase in spinach chloroplast envelopes: isolation by a calmodulin affinity chromatography. In Adv. Photosynth. Res., Proc. Int. Congr. Photosynth. 6th (Sybesma C, ed.), pp. II.6.607-10, Nijhoff, The Hague, Netherlands.

Schwitzguébel JP, Nguyen TD and Siegenthaler PA (1984). Are the phospholipid transfer proteins present in the stroma from higher plant chloroplasts ? In Structure, Function and Metabolism of Plant Lipids (Siegenthaler PA and Eichenberger A, eds.), pp. 299-302, Elsevier Science Publishers B.V.

Schwitzguébel JP, Nguyen TD and Siegenthaler PA (1985). Calmodulin is not involved in the regulation of exogenous NADH oxidation by plant mitochondria. Physiol. Plant. 63, 187-191.

Nguyen TD and Siegenthaler PA (1985). Proteins and polypeptides of envelope membranes from spinach chloroplasts. III. Purification and some properties of a Mg^{2+}-, Ca^{2+}- and calmodulin-stimulated ATPase. Biochim. Biophys. Acta 840, 99-106.

Les articles contenus dans ce dossier représentent l'essentiel du travail de thèse.

Le mémoire de thèse est déposé au Laboratoire de Physiologie végétale de l'Université de Neuchâtel (Professeur P.A. Siegenthaler), rue de Chantemerle 20, CH-2000 Neuchâtel (Suisse). Il peut être consulté en.tout temps sur place.

Experientia 38 (1982), 729

Proteins of spinach chloroplast envelopes

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Proteins of purified chloroplast envelopes, isolated according to Joyard and Douce (Physiol. Vég. *14*, 31, 1976), were separated by electrophoresis in a linear acrylamide gel gradient (SDS-PAGE) and by isoelectric focusing (PAGIF). SDS-PAGE resolved 37 polypeptides. The 2 major polypeptides (i.e. 54,000–56,000 and 15,000–16,000) of the envelope fraction were compared with the corresponding ones of the stroma and thylakoid fractions and identified as the subunits of the RuBP carboxylase or of the coupling factor by peptide mapping after limited proteolysis or by PAGIF in a 2nd dimension. Solubilization conditions of the envelope membranes for the PAGIF separation were studied in the presence of nonionic and ionic detergents. An adaptation of the method of Ames and Nikaido (Biochemistry *15*, 616, 1976), using solubilization in SDS and PAGIF in the presence of high concentration of Nonidet P40, gave the best separation and permitted to resolve the envelope fraction into 23–25 proteins.

Experientia 39 (1983), 651

Isolation of a Mg^{2+}-ATPase from spinach chloroplast envelopes using a calmodulin affinity column

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Envelopes from spinach chloroplasts contain at least 21 proteins and 37 polypeptides (Siegenthaler, P.A., and Nguyen, T.D., Biochim. biophys. Acta (1982), in press). On the other hand, the presence of calmodulin has been reported in spinach leaves (Simon, P., et al., Pl. Cell Rep. *1* (1982) 119). The aim of this investigation is to determine if some of these proteins have a specific affinity for calmodulin and what are their function.

Chloroplast envelope membranes were solubilized in triton X-100 and applied to a sepharose 4B calmodulin column in the presence of Ca^{2+} and phosphatidylcholine. The EGTA-eluted fraction contains 2 major proteins as revealed by isoelectric focusing and electrophoresis in native gels. These 2 proteins represent 3–4% of the total proteins. One of them shows an ATPase activity which is Mg^{2+}-dependent and activated by the addition of calmodulin and a mixture of triton X-100 and phosphatidylcholine.

Experientia 40 (1984), 614

Calcium, calmodulin and the oxidation of exogenous NADH by higher plants mitochondria

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Mitochondria from higher plants are able to oxidize exogenous NADH at high rates, via a dehydrogenase which is located at the outer surface of the inner membrane and not linked to the first energy-coupling site. The NADH oxidase from potato tubers mitochondria shows a specific dependence on calcium, in addition to a non-specific stimulation by cations. The electron flow from NADH to oxygen, exogenous cytochrome c or duroquinone, but not to ferricyanide, was strongly inhibited by EGTA and markedly stimulated by calcium. Calmodulin antagonists like chlorpromazine and phenothiazine were strong inhibitors of the NADH oxidase activity. This suggests that the effect of calcium on the activity of the NADH dehydrogenase could be mediated by calmodulin. However, the NADH duroquinone oxidoreductase, solubilized by Triton X-100, was not retained on either phenothiazine-Affi gel or calmodulin-Sepharose affinity columns. The possible direct or indirect regulation of NADH oxidation by calmodulin will be discussed.

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PROTEINS AND POLYPEPTIDES OF ENVELOPE MEMBRANES FROM SPINACH CHLOROPLASTS

I. ISOELECTRIC FOCUSING AND SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS SEPARATIONS

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Key words: Chloroplast envelope; Ribulosebisphosphate carboxylase; Isoelectric focusing; Polypeptide; Protein; (Spinach chloroplast)

Polypeptides of spinach chloroplast envelopes were separated by electrophoresis in an SDS-polyacrylamide gradient gel. At least 37 polypeptides were resolved; nine were prominent. Two (M_r 54000 and 16000) were also found in the stroma fraction and identified by peptide mapping and isoelectric focusing in the second dimension as the large and small subunits of ribulose-1,5-bisphosphate carboxylase. Proteins of the chloroplast envelope were also separated by isoelectric focusing. An adaptation of a previous method (Ames, G.F.L. and Nikaido, K. (1976) Biochemistry 15, 616–623), using solubilization in SDS and isoelectric focusing in the presence of a high concentration of Nonidet P-40, gave the best separation and resolved the envelope membranes into at least 21 proteins. The major band (p*I* 6.85) contained both subunits of the carboxylase and at least two additional polypeptides which corresponded to the prominent bands found in SDS gel electrophoresis of chloroplast envelopes.

Introduction

The two membranes that comprise the chloroplast envelope support a great variety of physiological functions that are sustained by the nature, amount and organization of the biochemical constituents [1–15]. Although the lipid and pigment composition of the envelope has been studied in detail [12], the function and organization of these components within the two membranes are not known. Surprisingly, the envelope contains little protein per unit mass compared to thylakoid membranes [12]. Electrophoretic patterns on SDS-polyacrylamide gels [4,5,9,10,12,13,16–23] show a series of high molecular weight bands ($M_r > 60000$) and four prominent polypeptides. The 29000 Da polypeptide has been demonstrated to be the phosphate translocator [20,21]. The 12000–14000 Da polypeptide was identified as the small subunit of ribulose-1,5-bisphosphate carboxylase [16] with anti-ribulose-1,5-bisphosphate carboxylase serum [22,23]. The identity of the 52000 Da polypeptide as the large subunit of ribulose-1,5-bisphosphate carboxylase, however, is controversial [16,22,23]. The isoelectric points of the proteins of chloroplast envelopes have not yet been reported, probably due to the difficulty of solubilizing the hydrophobic envelope proteins in nonionic detergents.

The aim of this investigation is first to obtain reproducible polypeptide separations of spinach chloroplast envelopes by SDS-polyacrylamide gel

Abbreviation: Tricine, *N*-tris(hydroxymethyl)methylglycine.

electrophoresis and to characterize the M_r of all polypeptides; secondly, to compare some of the polypeptides common to the envelope, stroma and thylakoid fractions with a similar M_r; and thirdly, to characterize the proteins of the chloroplast envelope by their isoelectric points. A preliminary report of these findings has been published [24].

Materials and Methods

Pure grade chemicals were supplied by Fluka with the exception of agarose, ammonium persulfate and Coomassie R-250 (Merck), urea (BDH), SDS (Serva), *Staphylococcus aureus* V_8 protease (Miles Laboratories), Ampholine (LKB), LMW electrophoresis calibration kit (Pharmacia) and the color reagent for the protein assay [25] which was purchased from Bio-Rad.

Chloroplast envelope preparation

Spinach (*Spinacia oleracea*) leaves were obtained from the local market. Envelopes were isolated from intact chloroplasts according to the method of Joyard and Douce [6,12]. The envelope, thylakoid and stromal proteins were estimated by the dye-binding method of Bradford [25] using bovine serum albumin as standard.

SDS electrophoresis

Electrophoresis was carried out in an Ortec model 4200 vertical slab gel ($100 \times 70 \times 3$ mm) system. Polyacrylamide gels were prepared essentially according to the method of Laemmli [26] with the following modifications: the separation gel consisted of a linear 8–14% (w/v) acrylamide gradient, accompanied by a 5–15% (w/v) sucrose gradient. The ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30:0.8. The catalyst concentrations were 0.02% (w/v) ammonium persulfate and a 0.04–0.02% (v/v) *N,N,N',N'*-tetramethylenediamine gradient in the gel. The gel was overlayered with water and electrode buffer, and kept at room temperature to complete the polymerization. On the following day, the 3% stacking gel was poured and the slot former inserted.

The samples for electrophoresis were solubilized in 50 mM Tris-HCl (pH 6.8), 2% (w/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol and incubated at 40°C for 30 min. An LKB 2103 power supply provided a constant current of 20 mA until the dye front reached the lower gel. The current was then increased to 30 mA until the tracking dye was about 1 cm from the bottom of the separating gel. Total electrophoresis time was 4–5 h.

The gel was fixed in 7% (v/v) acetic acid for 30 min (twice) and then stained in 0.25% Coomassie blue R-250, 25% (v/v) ethanol, 7% (v/v) acetic acid for 45 min. The gel was destained in 10% (v/v) ethanol and 7% (v/v) acetic acid until the background was clear, and finally stored in 7% acetic acid. The staining and destaining procedures were adapted from those of Weber and Osborn [27]. The destaining gel was scanned at 600 nm with a Zeiss-Disc ZK4 gel scanner. Calibration proteins were: phosphorylase *b* (94000); bovine serum albumin (67000); ovalbumin (43000); carbonic anhydrase (30000); soybean trypsin inhibitor (20100); α-lactalbumin (14400).

Proteolysis in the presence of SDS

SDS-polyacrylamide gel electrophoresis in the first dimension was performed as described above except that staining time was reduced to 15 min. The gel was destained by diffusion overnight. Bands of interest were excised from the gel with a razor blade, washed in 10 ml of distilled water for 30 min and incubated in 10 ml of 125 mM Tris-HCl (pH 6.8), 0.1% SDS for 30 min at room temperature with rotary shaking. After incubation, gel slices were placed on a stacking gel without slots and covered with a molten 1% agarose solution in 125 mM Tris-HCl (pH 6.8), 0.1% SDS. The separating gel for the second-dimension electrophoresis contained 15% acrylamide. After the gel was placed in the electrophoresis apparatus and overlayered with electrode buffer, a solution containing 100 μg protease in 125 mM Tris-HCl (pH 6.8), 0.1% SDS (w/v), 0.01% bromophenol blue (v/v), 20% glycerol (v/v) was layered on the hardened agarose according to Ref. 28. Electrophoresis was performed for 5 h at 10 mA. Digestion occurred directly in the stacking gel during the co-electrophoresis of the protease and the polypeptides. The electrophoresis was completed in the separating gel at 30 mA for 2 h and 40 mA for 1 h. Gel staining and destaining were carried out as described above.

Isoelectric focusing

Glass tubes (130 × 5 mm) were filled, leaving an adequate space for the sample, with gel solution containing 8 M urea, 5% (w/v) sucrose, 4.5% (w/v) acrylamide, 0.2% (w/v) *N,N'*-methylenebisacrylamide, 2% (w/v) Nonidet P-40, 2% (v/v) Ampholine (3.5–10), 0.06% (v/v) *N,N,N',N'*-tetramethylenediamine and 0.25% (w/v) ammonium persulfate.

Envelope membranes were solubilized in several concentrations of Nonidet P-40 (0.05–5%, v/v), corresponding to protein/Nonidet P-40 weight ratios of 1.3–0.03, for 30 min at 4, 20 and 40°C. Before loading onto the gel, sucrose was added to about 10% (w/v). Envelope membranes were also solubilized according to the method of Ames and Nikaido [29] in solutions of different SDS percentages (from 0.05 to 7.5%) and protein/SDS weight ratios (from 2.5 to 0.13) for 30 min at 4, 20 and 40°C. The solubilized proteins were diluted with 2 vol. of the following mixture (sample dilution buffer): 9.5 M urea, 2% (v/v) Ampholine (3.5–10) and 8% (w/v) Nonidet P-40. The Nonidet P-40/SDS ratio had to be greater than or equal to 8 [29].

The prepared chloroplast envelope proteins (200 μg) were loaded on each rod gel. SDS/Nonidet P-40-treated samples were overlayered with 50 μl of a solution containing 1 M urea, 2% (v/v) Ampholine (3.5–10), 2% (w/v) Nonidet P-40; Nonidet P-40-treated samples were covered with 50 μl containing 1% (w/v) Nonidet P-40, 2% (v/v) Ampholine (3.5–10), 5% (v/v) glycerol. Electrode solutions were 0.15 M ethanolamine for the upper chamber (cathode) and 0.1 M HCl for the lower chamber (anode) [30]. Electrofocusing was performed at room temperature with a Bio-Rad Model 155 electrophoresis system, at 250 V for 15 h and 500 V for 1 h. At the end of the run, gels were fixed for 30 min in 7% (v/v) acetic acid (twice) and then equilibrated for 30 min in 25% (v/v) ethanol, 7% (v/v) acetic acid and stained for at least 45 min in the staining solution. The gel was destained in the equilibrating solution and scanned at 600 nm. A blank, unstained gel was sliced into pieces of 5 mm which were eluted in 1 ml of distilled water for 4 h and the pH of the eluted gel sections was determined [30].

Results and Discussion

SDS-polyacrylamide gel electrophoresis

The separation of envelope, thylakoid and stromal polypeptides, obtained in a linear acrylamide gel gradient, is shown in Fig. 1. The purified envelope fraction contains at least 37 polypeptide bands (Fig. 1E). A densitometric tracing of the gel (Fig. 2) showed nine predominant polypeptides (99 000, 88 000, 75 000, 65 000, 54 000, 34 000, 29 000, 27 000 and 16 000 Da) and a large number of minor polypeptides which were present either in small amounts (170 000, 148 000, 137 000, 120 000, 70 000, 45 000, 42 000–38 000, 36 000) or were close to another band. The latter polypeptides appeared as shoulders or as small peaks depending on the envelope preparations (arrows in Fig. 2). Under the selected conditions, the apparent M_r of each polypeptide was reproducible: the standard deviation ranged from 300 to 1800

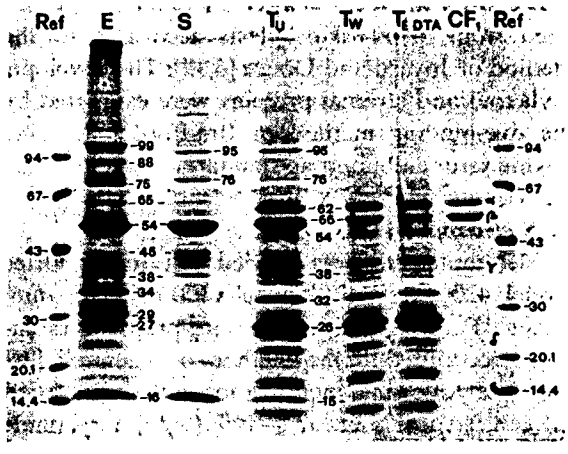

Fig. 1. Electrophoretic separation of polypeptides from different chloroplast fractions: E, envelopes (50 μg proteins); S, stroma (40 μg); Tu, unwashed thylakoids (40 μg); T_w, once-washed thylakoids (40 μg); T_{EDTA}, once-washed thylakoids treated with 0.75 mM EDTA (40 μg); CF_1, supernatant of EDTA-treated thylakoids (10 μg); Ref, standard proteins (12 μg). Apparent M_r values are indicated in kDa. α (62 kDa), β (56), γ (38), δ (23), ε (15) correspond to the subunits of CF_1. The different chloroplast fractions were obtained in the same step sucrose gradient according to Refs. 6 and 12. Unwashed thylakoids were collected, diluted with a suspension medium containing 0.3 M sucrose, 10 mM Tricine-NaOH (pH 7.6) and centrifuged at 17000 × *g* for 5 min. The pellet (washed thylakoids) was then resuspended in the same medium to about 2 mg chlorophyll/ml.

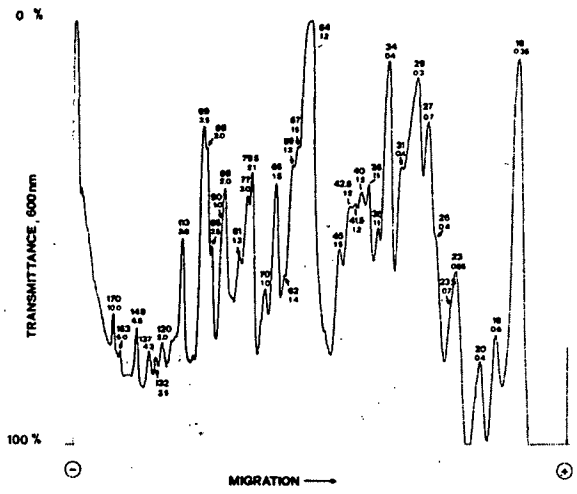

Fig. 2. Densitometric tracing of envelope polypeptides (E in Fig. 1) separated by SDS-polyacrylamide gradient gel electrophoresis. Peaks and shoulders are characterized by their apparent M_r and standard deviation ($n = 7-10$).

Da for polypeptides of 16000–70000 Da. Above this value the standard deviation increased (Fig. 2). Unlike previous reports [5,9,10,16,18,19,21–23] it was now possible to assign an M_r to all the polypeptides, including the minor ones.

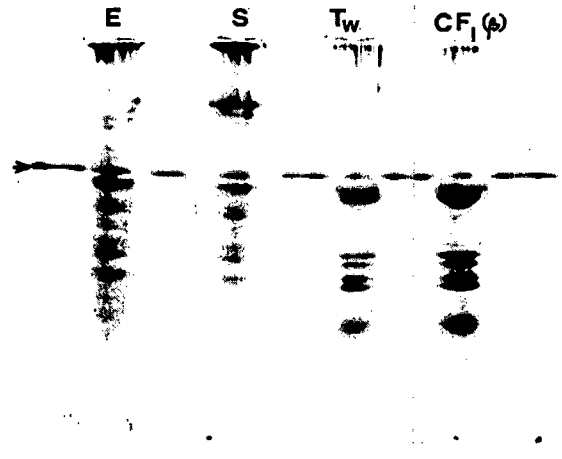

Fig. 3. Peptide mapping of the 54000–56000 Da polypeptides from the envelope (E), stromal (S), washed thylakoid (T_w) fractions and from the supernatant of the EDTA-treated thylakoids (CF_1). Depending on the fraction used, one to five bands (corresponding to 54000–56000 Da polypeptide) obtained in the first dimension were pooled on top of the second-dimension gel and the *S. aureus* V_8 protease (100 μg) was overlayered as described in Materials and Methods. The position of the protease in the gel is indicated by the arrow.

When the polypeptides of the stromal and thylakoid fractions were separated electrophoretically in a linear acrylamide gel gradient (Fig. 1, S and T_w), the densitometric tracing indicated that these two fractions contained at least 37 and 24 polypeptides, respectively (not shown). The electrophoretic pattern of envelope and stromal fractions (Fig. 1E and S) consisted of at least six major band groups corresponding to similar apparent M_r polypeptides (75 000–76 000, 65 000, 54 000, 38000–45000, 27000 and 16000). The electrophoretic pattern of envelope and thylakoid fractions (Fig. 1E and T_w) also contained polypeptides having similar apparent M_r (54000–56000, 38000, 32000–34000 and 26000–27000).

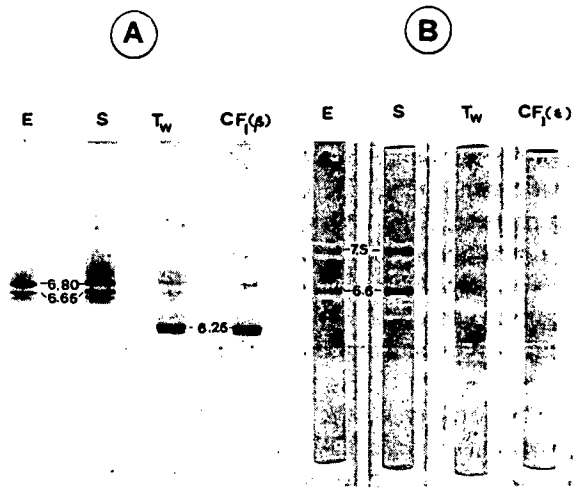

Fig. 4. Isoelectric focusing separation of the 54000–56000 (A) and 15000–16000 Da (B) polypeptides from different fractions obtained in a first-dimensional SDS-polyacrylamide gel electrophoresis. In this particular experiment, solubilization of the different fractions (E, envelope; S, stroma; T_w, washed thylakoids; CF_1, coupling factor: supernatant of the EDTA-treated thylakoids) was carried out as described in Materials and Methods. After the electrophoretic separation in the first dimension, the polypeptides concerned were visualized by a solution of 0.25 M KCl [32] for 10–15 min. The whitish and opaque bands were cut out, and soaked twice in 1 ml H_2O for 10 min. After the removal of water, the gel slices were crushed, macerated and eluted in 400 μl of 50 mM Tris-HCl (pH 6.8) for 1 h under gyratory motion. Then solid urea, Nonidet P-40 and Ampholine (3.5–10) were added to about 6 M, 4% (w/v) and 2% (v/v), respectively. The entire suspensions were loaded on top of the rod gels (80×5 mm) and overlayered with 1% (v/v) Ampholine (3.5–10), 1% (w/v) Nonidet P-40 and 10% (v/v) glycerol. Other conditions as in Materials and Methods. Figures correspond to p*I* values.

To test if the 54000–56000 Da polypeptides, common to all three fractions, had exactly the same electrophoretic mobility, an appropriate amount of each of the three initial samples was loaded on a gel to obtain very thin bands (for the 54000–56000 Da polypeptides). The results of six experiments confirmed that the electrophoretic mobility of the 54000 Da polypeptides from the envelope and stromal fractions was identical and that the apparent M_r of the corresponding polypeptide from the thylakoid fraction was always slightly higher (56000 Da). In addition, a partial digestion of the 54000 Da polypeptide with protease from *S. aureus* V_8 showed that the peptide fragments from envelope and stromal fractions were identical while the pattern from the thylakoid polypeptide was different (Fig. 3E, S and T_w). This was further demonstrated by the behavior of these polypeptides in a second dimension, i.e., in a pH gradient polyacrylamide gel. Isoelectric focusing separations of the envelope and stromal polypeptides showed a similar pattern consisting of at least 3 bands (Fig. 4A, E and S). The corresponding pattern of the thylakoid polypeptides (Fig. 4A, T_w) was different (at least two major and approximately five minor bands of different p*I* values) but was similar to the pattern of the 56000 Da polypeptide from the β-subunit of CF_1, (CF_1 in Fig. 4A).

The identity of the 54000 Da polypeptide is controversial [16,22]. We have demonstrated that this polypeptide corresponds to the large subunit of ribulose-1,5-bisphosphate carboxylase in three different ways. First, the electrophoretic mobility of this envelope polypeptide corresponds exactly to that of the main band of the stroma (Fig. 1E and S). Secondly, the peptide maps of both envelope and stromal bands are identical (Fig. 3E and S). Thirdly, isoelectric focusing in the second dimension reveals an identical pattern (Fig. 4A, E and S). These results are consistent with those of Pineau et al. [22] and Joyard et al. [23] who have excluded the possibility of contamination of the envelope vesicles by the stroma. Since the large subunit of the carboxylase is synthesized inside the chloroplast and consequently does not cross the envelope like the small subunit, it is difficult to explain its presence in the envelope fraction. The simplest explanation is that the holoenzyme of the carboxylase has an affinity for envelope membranes and is not removed by the procedures used to isolate envelopes. Association of the carboxylase with the envelope might also be of physiological significance: the assembly of both subunits may be an envelope event.

Since the enzymatic digestion was limited with polypeptides having an M_r less than 20000, the M_r 15000–16000 polypeptides from the envelope and stromal fractions (Fig. 1) were subjected to isoelectric focusing in a second dimension. Fig. 4B shows that the 15000 Da polypeptides were resolved into identical bands. This polypeptide can be assigned to the small subunit of the carboxylase [16,22,23]. Moreover, the presence of several bands in Fig. 4B may be due to isofocusing variants as was observed for the small subunit of the carboxylase in *Pisum sativum* [31]. If the M_r of the spinach small subunit precursor is similar to that of *P. sativum* [33] and *Chlamydomonas* [34], the 20000 Da polypeptide found in our gel (Figs. 1 and 2) might be the precursor itself. Similarly, the 34000 Da polypeptide (Fig. 2) which probably corresponds to the 33000 and 32000 Da polypeptides described by Pineau and Douce [16] and Ellis [35], respectively, might be the precursor of the major polypeptide of the light-harvesting chlorophyll *a/b*-protein complex (see Discussion in Ref. 36). The phosphate translocator (M_r 29000) which is as yet the best characterized polypeptide in chloroplast envelopes [20,21] was well defined in our gel (Figs. 1 and 2).

The nature of the 54000–56000 Da band encountered in thylakoid membranes was also investigated by comparing the electrophoretic separation of unwashed, washed and EDTA-extracted thylakoids as shown in Fig. 1. A single washing of thylakoids (Fig. 1, cf. T_u and T_w) resulted in the disappearance of the lighter portion of the double band (54000 Da) and a significant loss of the 15000 Da polypeptide band. Thus, thylakoid washing(s) eliminated the two subunits of the ribulose-1,5-bisphosphate carboxylase and revealed the β- and ε-subunits of the coupling factor (Fig. 1, T_u, T_w, T_{EDTA} and CF_1; see also Ref. 36) which were identified by peptide mapping (Fig. 3, T_w and CF_1) and isoelectric focusing in the second dimension (Fig. 4B, T_w and CF_1). The isoelectric focusing pattern of the 16000 Da polypeptides from washed thylakoids and CF_1 were completely

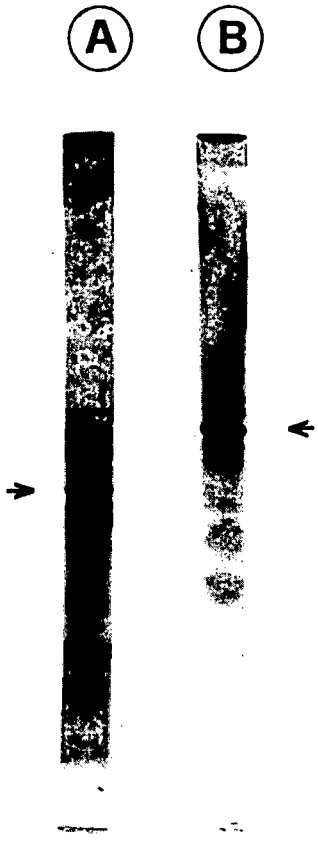

Fig. 5. Separation of chloroplast envelope proteins by polyacrylamide gel isoelectric focusing. (A) Membranes (200 μg proteins) were solubilized at 40°C for 30 min in 2% Nonidet P-40 (weight ratio of protein to detergent, 1:15). (B) Membranes (200 μg proteins) were solubilized at 40°C for 30 min in 0.1% SDS (weight ratio of protein to detergent, 1:0.75). For other conditions see Materials and Methods.

different from that of the envelope and stromal fractions (Fig. 4B). Due to the small amount of 16000 Da polypeptide available in the first-dimension separation of washed thylakoids and CF_1 (T_w and CF_1 in Fig. 1) it was not possible to demonstrate unequivocally their identity.

Polyacrylamide gel isoelectric focusing

In the presence of nonionic detergents such as Nonidet P-40 or Triton X-100, the best separations (thin bands, minimum of streakings) were obtained by incubating chloroplast envelopes in 2% Nonidet P-40, at a protein/Nonidet P-40 weight ratio of 0.07 and at 40°C for 30 min (Fig. 5A). Nonionic detergents, however, do not permit complete solubilization of membrane proteins [30,37]. Thus, we have adopted the technique proposed by Ames and Nikaido [29] involving SDS solubilization followed by isoelectric focusing in the presence of a high concentration of Nonidet P-40. The resolution of the envelope proteins was good (not shown) for all concentrations of SDS between 0.05 and 0.2% in the solubilization medium and for a protein/SDS weight ratio varying between 2.5 and 0.7. At higher SDS concentrations it was found that the staining intensity of the acidic proteins decreased and the alkaline region of the gel became darker. Solubilization of chloroplast envelopes in 0.1% SDS and a protein/SDS weight ratio of 1.33 for 30 min at 40°C resulted in

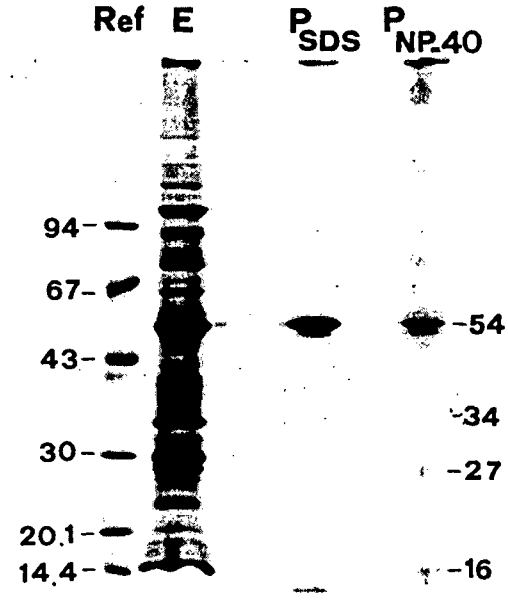

Fig. 6. Electrophoretic separations in SDS-polyacrylamide gel (second dimension) of proteins obtained by isoelectric focusing (first dimension). The proteins obtained by isoelectric focusing in the first dimension (arrows in Fig. 5) were revealed by staining, cut off with a razor blade, washed for 30 min in water, then equilibrated for 30 min in 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS. The slices were placed on the stacking gel of the second dimension and sealed with 1% agarose in the same Tris buffer. Other conditions as in Materials and Methods, Ref, standard proteins (kDa) as in Fig. 1; E, control envelope polypeptides; P_{SDS} and P_{NP-40}, SDS-electrophoretic separations of the main proteins focused after solubilization in SDS/Nonidet P-40 and Nonidet P-40, respectively.

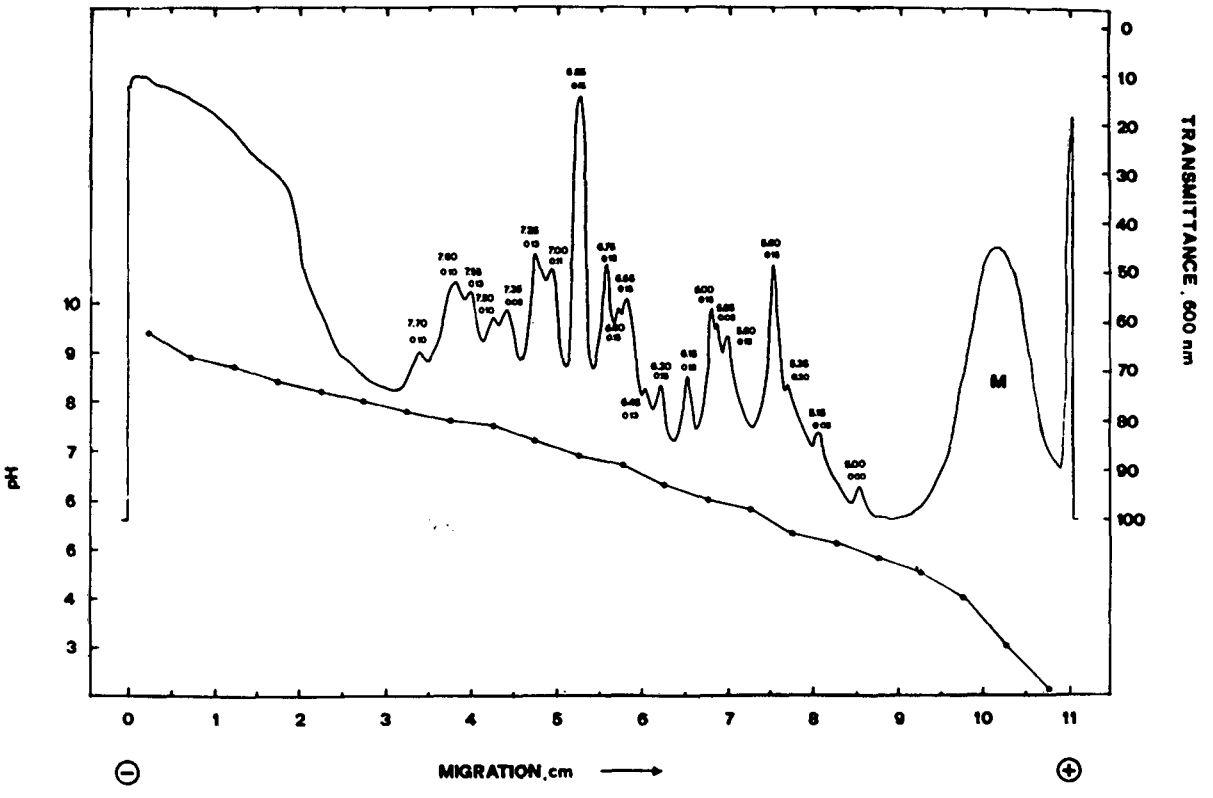

Fig. 7. Densitometric tracing of envelope proteins separated by polyacrylamide gel isoelectric focusing. The chloroplast envelopes were solubilized in SDS (0.1%)/Nonidet P-40 (5.3%) at a protein/SDS weight ratio of 1.33. Peaks and shoulders are characterized by their p*I* (see pH gradient) and standard deviation (n = 5–20). The M band contains detergent/carotenoid micelles.

the best isoelectric focusing separation (Fig. 5B).

Since SDS is a denaturing detergent it was important to verify that envelope proteins had electrofocusing behavior after addition of Nonidet P-40 to the SDS-solubilization medium, identical to those solubilized in Nonidet P-40 alone. As shown in Fig. 6, the main band obtained by isoelectric focusing after solubilization with Nonidet P-40 alone or SDS/Nonidet P-40 (arrows in Fig. 5) was further separated by SDS-polyacrylamide gel electrophoresis into at least five identical polypeptides. It is noteworthy that the two proteins focusing at the same pH gave rise to polypeptides having M_r values of 54000 (accompanied by a satellite band having a smaller M_r), 34000, 27000 and 16000. The 54000 and 16000 Da polypeptides belong to ribulose-1,5-bisphosphate carboxylase as attested by the SDS gel electrophoretic separation of control envelope proteins (Fig. 6E). These results show quite clearly that the native charges of the chloroplast envelope proteins were preserved and identical after both solubilizing treatments.

As shown in Fig. 7, the isoelectric focusing separation of spinach chloroplast envelopes displayed at least 21 proteins, ranging from p*I* 5 to 8. The number of native proteins was of course less than that of polypeptides. The scanning profile revealed only one prominent protein having a p*I* of 6.85. As shown previously (Fig. 6), it corresponded mainly to ribulose-1,5-bisphosphate carboxylase (M_r 54000 and 16000). It is significant that the p*I* of one of the variants of the small carboxylase subunit from *Chlamydomonas reinhardii* was found to be 6.85 [38]. Six proteins with the following p*I* values (7.25, 7.00, 6.75, 6.55, 6.00 and 5.60) were less intensely stained. The other bands, although minor, were always encountered in the scanning

profile. The dark region in the basic part of the gel (probably due to the binding of SDS to the positively charged ampholytes) might mask the presence of proteins. However, when Nonidet P-40 was used in the solubilization medium instead of SDS/Nonidet P-40, this region of the gel became clearer and did not show the presence of any proteins. The scanning profiles of the envelope proteins solubilized by the two methods were very similar (not shown) but the bands were better resolved with the combined SDS/Nonidet P-40 detergents. This is probably due to the pronounced hydrophobic character of this type of membrane [12,23]. These optimal conditions allowed us to obtain reproducible isoelectric focusing separations; the standard deviation of the p*I* values did not exceed 0.15 pH unit (Fig. 7).

Acknowledgements

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Proteins and polypeptides of envelope membranes from spinach chloroplasts

Properties of a membrane-bound ATPase

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Spinach chloroplasts are known to contain calmodulin and to display an envelope-bound ATPase activity. This activity, stimulated by 0.15 mM Ca^{2+} and 5 mM Mg^{2+}, is further enhanced by calmodulin. The apparent K_m for ATP was 0.55 mM. The enzyme was especially sensitive to NH_4VO_4, $SbCl_3$, $LaCl_3$ and oligomycin. An attempt to isolate the ATPase by calmodulin–Sepharose affinity chromatography was successful. The EGTA-eluted fraction contained 2 proteins out of the 21 proteins separated previously by isoelectric focussing [(1983) Biochim. Biophys. Acta 722, 226–233] and exhibited an ATPase activity.

ATPase *Chloroplast envelope* *Spinach* *Calmodulin* *Cation activation*

1. INTRODUCTION

A Mg^{2+}-dependent ATPase, insensitive to *N,N'*-dicyclohexylcarbodiimide (DCCD), is associated with chloroplast envelopes [1]. This enzyme has a greater affinity for Mn^{2+} than for Mg^{2+} [2]. More recently, oligomycin was found to inhibit partially the envelope ATPase activity [3].

In plant tissues, calmodulin modulates the activity of at least 3 enzymes: NAD^+ kinase, Ca^{2+}-ATPase and quinate: NAD^+ oxidoreductase [4]. Calmodulin is found in spinach [5], pea [6] and wheat [7] leaves and is present mainly in the cytosol (90%) and to a lesser extent in mitochondria (5–9%), chloroplasts (1–2%) and the microsomal fraction (<1%) [7]. In chloroplasts, calmodulin appears to be confined in the stroma [6]. Calmodulin antagonists such as chlorpromazine or phenothiazine inhibit electron transport in photosystem II of spinach chloroplasts [8] and the proton gradients associated with photophosphorylation [9].

The aim of this study was to investigate the properties of the chloroplast envelope-bound ATPase, namely the effect of various ions and calmodulin on its activity. Furthermore, an attempt was made to isolate a protein which has a specific affinity for calmodulin and exhibits an ATPase activity among the 21 chloroplast envelope proteins separated by isoelectric focussing [10].

2. MATERIALS AND METHODS

2.1. *Preparation of envelopes*

Deveined spinach leaves (700–800 g) were homogenized in 1.5 l grinding medium (25 mM Tricine–NaOH, pH 7.8, 300 mM sucrose, 0.1% defatted bovine serum albumin, 1 mM phenylmethanesulfonyl fluoride: PMSF) in a 1 gallon Waring Blendor for 5 × 1 s at 4°C. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 1500 × *g* for 5 min. The pellets of crude chloro-

This paper is dedicated to Professor Claude Favarger, Director of the Institute of Botany, University of Neuchâtel, Switzerland, in honor of his 70th birthday

This paper is the second of a series (see [10])

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plasts were resuspended in the grinding medium, then layered on a 40% Percoll solution having the same composition as the grinding medium (20 ml suspension/15 ml Percoll 40%) and centrifuged at 2000 × *g* for 5 min [11]. The supernatant was discarded by aspiration and intact chloroplasts were washed once with the grinding medium and spun down at 2000 × *g* for 10 min. The intact and purified chloroplasts were lysed by osmotic shock in 10 mM Tricine–NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM PMSF. Thylakoids were partially removed by a centrifugation at 12000 × *g* for 10 min; 18 ml of the resulting yellow-brown supernatant were layered on the top of a two-step sucrose gradient: 1.0 M (10 ml) and 0.6 M (10 ml) sucrose in 10 mM Tricine–NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM PMSF. A centrifugation at 95000 × *g* (R_{max}) for 1 h revealed a yellow band (envelope fraction) at the sucrose interface. The envelopes were collected, diluted 4–5-fold with 10 mM Tricine–NaOH (pH 7.8) and sedimented at 122000 × *g* (R_{max}) for 45 min.

2.2. *ATPase assay*

The ATPase activity was assayed in a 250 μl reaction mixture which contained 50 mM Tris–HCl (pH 7.8), 300 mM sucrose, 10–15 μg envelope protein. Concentrations of ATP, $MgCl_2$ and $CaCl_2$ are indicated in the legends of the figures. When $CaCl_2$ was used, 1 mM EGTA was added to the reaction mixture and free Ca^{2+} concentrations were calculated from the EGTA: Ca^{2+} ratio as in [12]. No correction was made for the ATP-Ca binding. Assays were routinely made in duplicate with adequate controls. After preincubation at 37°C for 10 min, the reaction was started by the addition of ATP. After 15 min at 37°C, the assays were stopped with 50 μl of 60% trichloroacetic acid. Under these conditions, the enzyme kinetics were linear up to 45 min (not shown). Precipitated proteins were removed by centrifugation (1 min in a Beckman Microfuge B) and P_i was measured in the supernatant as in [13]. A P_i calibration curve was made in the presence of 10% trichloroacetic acid.

2.3. *Other methods*

The isolation of the partially purified ATPase from chloroplast envelopes was carried out essentially as in [14], using calmodulin–Sepharose affinity chromatography. Protein was determined as in [15]. Calmodulin was purified from bovine brain [16].

3. RESULTS AND DISCUSSION

3.1. *Effect of ATP*

Fig.1 shows that the envelope-bound ATPase activity as a function of ATP concentrations followed Michaelis–Menten kinetics with an apparent K_m-value for ATP of 0.55 mM as determined by the double-reciprocal plot of $1/V$ *vs* $1/S$ (inset of fig.1). Compared to the value reported earlier [2], our enzyme had a greater affinity for ATP by a factor of 1.4. A basal ATPase activity, varying between 60–80 nmol P_i released · mg protein^{-1} · min^{-1} was associated with chloroplast envelopes (see control in fig.2). This activity was much higher than that (about 3 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$) found in [2]. This basal activity apparently did not depend on divalent cations which could be externally bound to envelope vesicles since it was EDTA-insensitive.

3.2. *Effect of ions, buffers and inhibitors*

The ATPase activity was stimulated by divalent cations with maximal rates at 0.15 mM $CaCl_2$ and 5–10 mM $MgCl_2$ (fig.2). It is noteworthy that

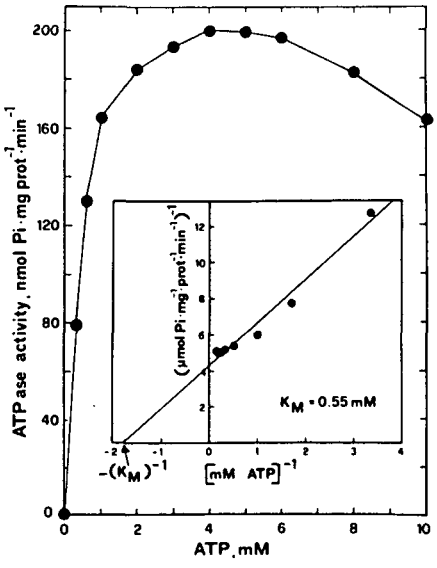

Fig.1. ATP concentration dependence of Mg^{2+}-stimulated envelope-bound ATPase activity. The reaction mixture contained 5 mM $MgCl_2$ and ATP as indicated. A double-reciprocal plot of $1/V$ *vs* $1/S$ is shown in the inset ($r = 0.98$).

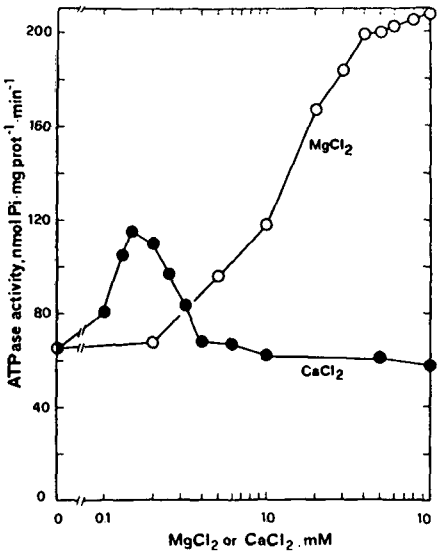

Fig.2. Stimulation of envelope-bound ATPase activity by $MgCl_2$ and $CaCl_2$. The reaction mixture contained 4 mM ATP and salts at the concentrations indicated.

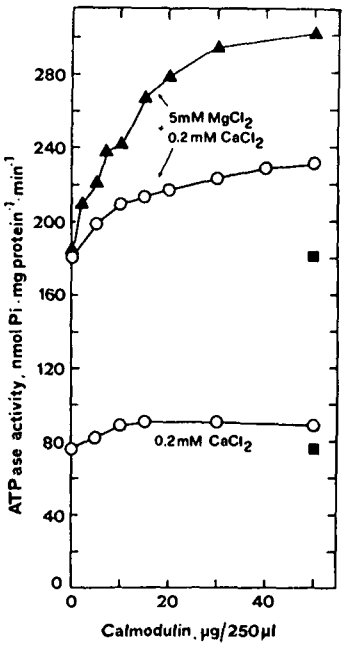

Fig.3. Stimulation of envelope-bound ATPase activity by calmodulin in the presence of $CaCl_2$ alone or $MgCl_2$ + $CaCl_2$. The reaction mixture contained 4 mM ATP and calmodulin at the concentrations indicated. (▲) spring spinach; (○) summer spinach; (■) effect of 80 µM chlorpromazine.

above 0.4 mM, $CaCl_2$ did not stimulate the enzyme activity. In the presence of 5 mM $MgCl_2$, 0.01 mM $CaCl_2$ caused a 30% increase in the activity (not shown). $MnCl_2$ (5 mM, pH 7.8) also stimulated the ATPase activity but to a lesser extent than $MgCl_2$ (about 2/3 of the stimulation observed with $MgCl_2$). A survey of the effect of other salts showed that $CuSO_4$ (5 mM) and $ZnSO_4$ (5 mM) in the absence of $MgCl_2$, KCl (50 mM), NaCl (50 mM), NH_4Cl (50 mM) in the presence of 5 mM $MgCl_2$, only slightly activated the ATPase. However, $SbCl_3$ (1 mM) and $LaCl_3$ (1 mM) in the presence of 5 mM $MgCl_2$ inhibited 50% of the activity. Among 3 different Mg salts ($MgCl_2$, $MgSO_4$, $Mg(NO_3)_2$) tested, the chloride salt was the most efficient on the enzyme activity. Comparison of ATPase activity at pH 7.8 showed that the specific activity with the standard buffer (50 mM Tris–HCl) was up to 25% higher than that in 50 mM Tricine–NaOH. Among several inhibitors tested (NaN_3, oligomycin, ouabain, NH_4VO_4, DCCD, EGTA) only oligomycin (5 µg/250 µl) and NH_4VO_4 (0.2 mM) inhibited the activity by 25 and 98%, respectively.

Thus, the present envelope-bound ATPase exhibited several differences with that described in [2]: (i) The ATPase activity did not depend strictly on divalent cations; (ii) $MgCl_2$ ($\geq$5 mM) and to a lesser extent $MnCl_2$ stimulated the activity; (iii) $CaCl_2$ was found to be an activator of the enzyme at low concentrations (0.15 mM). In addition to the inhibitory effect of oligomycin, which was already described [3], we found that NH_4VO_4, $LaCl_3$ and $SbCl_3$ were potent inhibitors of the envelope-bound ATPase.

3.3. *Effect of calmodulin*

In the presence of both $MgCl_2$ and $CaCl_2$, calmodulin further stimulated the activity (fig.3). The extent of the stimulation was 63% with spring and 28% with summer spinach. This stimulation was not only $CaCl_2$- but also $MgCl_2$-dependent. An antagonist of calmodulin such as chlorpromazine, suppressed completely the effect of calmodulin, as shown in fig.3. It is the first time that a chloroplast envelope-bound enzyme is found to be stimulated by calmodulin.

3.4. *Partial purification of an envelope ATPase*

Since calmodulin was found to enhance the activity of the envelope-bound ATPase, we postulated

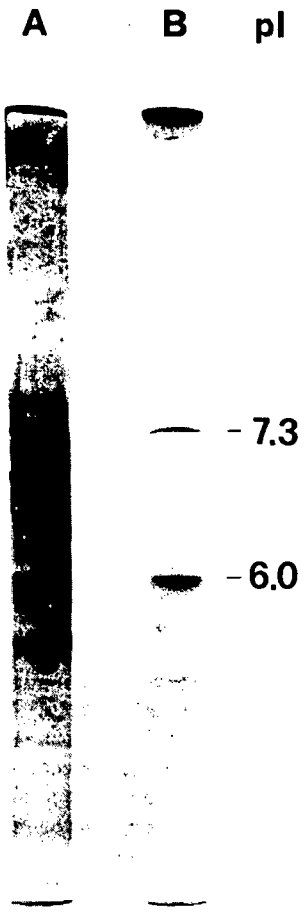

Fig.4. Separation of proteins by isoelectric focussing. (A) chloroplast envelope proteins. (B) EGTA-fraction eluted from the calmodulin–Sepharose column. Conditions for isoelectric focusing were as in [10].

that the enzyme might have a specific affinity for calmodulin. We attempted therefore to retain specifically the ATPase on a calmodulin–Sepharose affinity column in the presence of calcium. Fig.4 shows the separation by isoelectric focusing of the total envelope proteins and of the EGTA-eluted fraction which contained only two proteins. This latter fraction displayed an ATPase activity which was also stimulated by Ca^{2+}, Mg^{2+} and calmodulin (not shown).

In conclusion, the fact that the envelope-bound ATPase is sensitive to Ca^{2+} at low concentrations, to Mg^{2+} at much higher concentrations and to calmodulin opens interesting perspectives in the understanding of the regulation of photosynthesis. Due to its special properties this enzyme might well modulate ion, metabolite and/or protein exchanges between the chloroplast and the cytosol.

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A (Mg^{2+}-Ca^{2+})-STIMULATED ATPase IN SPINACH CHLOROPLAST ENVELOPES: ISOLATION BY CALMODULIN AFFINITY CHROMATOGRAPHY

T.D. NGUYEN/P.A. SIEGENTHALER

1. INTRODUCTION

A Mg^{2+}-dependent ATPase, insensitive to N,N'-dicyclohexylcarbodiimide is associated with chloroplast envelopes (Douce et al. 1973). This enzyme has a greater affinity for Mn^{2+} than for Mg^{2+} (Joyard and Douce 1975). The partial inhibition of the envelope ATPase by oligomycin has suggested that this enzyme may play a role in mediating H^+ efflux and K^+ uptake in chloroplasts (Maury et al. 1981).

Calmodulin is present in leaf tissues (Watterson et al. 1980; Muto, 1982; Jarrett et al. 1982) mainly in the cytosol (90%) and to a lesser extent in mitochondria (5-9%), chloroplasts (1-2%) and the microsomal fraction (1%). In plant tissues, calmodulin modulates the activity of at least three enzymes : NAD^+ kinase, Ca^{2+}-ATPase and quinate : NAD^+ oxidoreductase (Marmé 1982). In chloroplast, calmodulin seems to be confined in the stroma (Jarrett et al. 1982). Recently, calmodulin antagonists (chlorpromazine, phenothiazine) were shown to inhibit photochemical reactions in spinach chloroplasts (Barr and Crane 1982). It is likely that calmodulin is involved in the regulation of photosynthesis (Jarrett et al. 1982) by interacting with NAD^+ kinase (maybe with an ATPase), if not from the stroma at least from chloroplast envelope.

The first aim of this study was to investigate the properties of the chloroplast envelope-bound ATPase, namely the effect of Mg^{2+}, Ca^{2+} and calmodulin on its activity. The second aim was to isolate, out of the 21 chloroplast envelope proteins separated by isoelectric focusing (Siegenthaler and Nguyen, 1983), a protein which had a specific affinity for calmodulin and which displayed an ATPase activity. Finally, the properties of the two ATPases were compared.

2. MATERIALS AND METHODS

Spinach chloroplast envelopes were prepared according to Douce et al. (1973) and resuspended in buffer A (50 mM Tris-HCl, pH 7.8, 300 mM sucrose). Envelopes were solubilized with 5 mg Triton X-100/mg protein at 4°C for 15 min, then 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ were added. The unsolubilized material was pelleted at 100 000 x g for 30 min at 4°C. The supernatant containing the solubilized ATPase was loaded onto a calmodulin-Sepharose column which was equilibrated with buffer B (buffer A with 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05% Triton X-100 and 1 mM 2-mercaptoethanol). Bovine brain calmodulin (100 mg) was purified essentially according to Gopalakrishna and Anderson (1982) and was coupled to 7 g CNBr-activated Sepharose 4B [see Pharmacia instructions]. The column was washed first with buffer B and then with buffer B + 0.5 M NaCl. Proteins were eluted with buffer B without $CaCl_2$ but with 1 mM EGTA. The ATPase activity was assayed by measuring the release of Pi according to Lebel et al. (1978). Proteins were estimated according to Bradford (1976).

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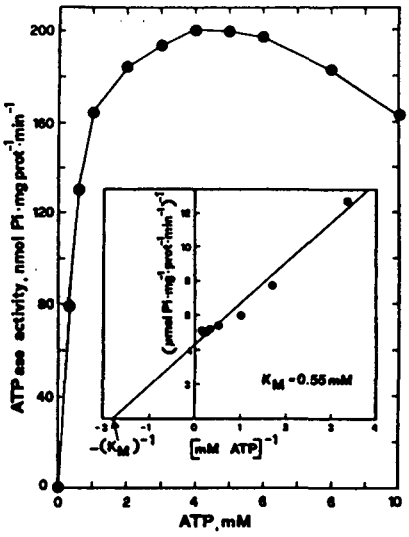

Fig. 1. ATP dependence of envelope-bound ATPase activity. The reaction mixture contained 50 mM Tris-HCl (pH 7.8), 300 mM sucrose, 5 mM $MgCl_2$, envelopes (10-15 µg protein/250 µl) and ATP as indicated. Incubation: 15 min, 37°C.

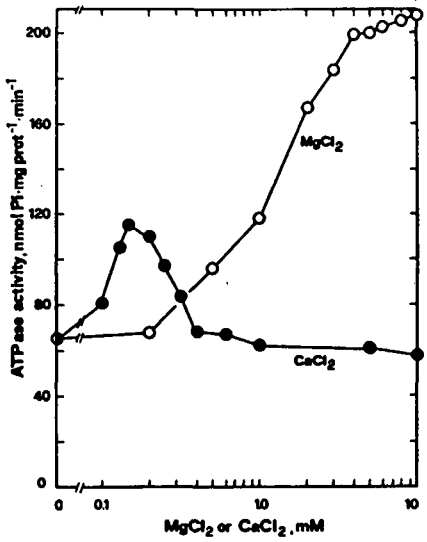

Fig. 2. Stimulation of envelope-bound ATPase activity by $MgCl_2$ and $CaCl_2$. Conditions as in Fig. 1 but ATP = 4 mM and concentrations of cations as indicated.

RESULTS AND DISCUSSION

The envelope-bound ATPase activity as a function of ATP concentration followed Michaelis-Menten kinetics with an apparent K_M value for ATP of 0.55 mM as determined by the double-reciprocal plot of 1/V versus 1/S (inset of Fig. 1). Compared to the value found by Joyard and Douce (1975), our enzyme had a greater affinity for ATP by a factor of 1.4. A basal ATPase activity, EDTA-insensitive, was associated with chloroplast envelopes. It was stimulated by divalent cations with maximal rates at 0.15 mM $CaCl_2$ and 5 to 10 mM $MgCl_2$ (Fig. 2). In the presence of Ca^{2+} and Mg^{2+}, calmodulin further stimulated the activity (28-63%, see Table I). The enzyme was sensitive to oligomycin, $LaCl_3$ and NH_4VO_3.

Envelope proteins which were bound to the calmodulin-Sepharose column in the presence of calcium and eluted by EGTA, showed two bands on native and isoelectric focusing gels (pIs 7.3 and 6.0). This EGTA-fraction contained an ATPase, the properties of which were quite similar to those of the envelope-bound enzyme. The K_M (ATP) was 0.45 mM (Fig. 3) and maximal rates were at 0.05 mM $CaCl_2$ and 1 mM $MgCl_2$ (Fig. 4). In the presence of both cations, calmodulin caused a 40% stimulation of the ATPase activity (Table I).

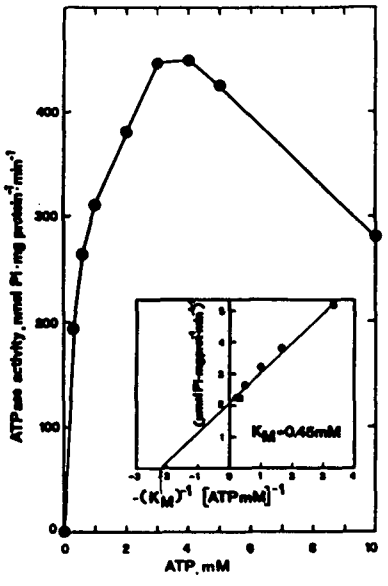

Fig. 3. ATP dependence of partially purified ATPase activity. Conditions as in Fig. 1 but $MgCl_2$ = 1 mM, ATPase (1-3 µg protein/250 µl), 60 min.

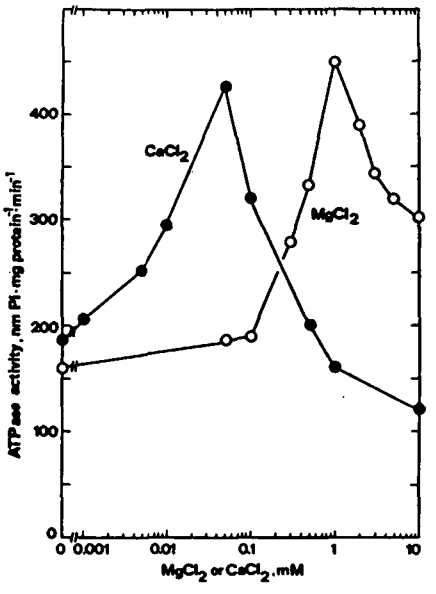

Fig. 4. Stimulation of partially purified ATPase activity by $MgCl_2$ and $CaCl_2$. Conditions as in Fig. 3 but ATP = 4 mM.

TABLE I. Activation of the membrane-bound and partially purified ATPase from spinach chloroplast envelopes

	Specific activity : nmol Pi·mg $Prot^{-1}$·min^{-1}		
	Membrane bound ATPase		Partially purified ATPase
Control[a]	180[c]	185[d]	420[c]
+ calmodulin[b]	231[c]	301[d]	586[c]

[a]Conditions as in Fig. 3; [b]200 µg/ml for the envelope-bound and 40 µg/ml for the purified ATPase; [c]Summer spinach; [d]Spring spinach.

In conclusion, the use of calmodulin-Sepharose affinity chromatography enabled to isolate quickly a protein fraction enriched in ATPase activity. This partially purified enzyme had properties which were similar to those of the envelope-bound ATPase, in particular both were stimulated by Ca^{2+} and Mg^{2+} ions and by calmodulin. In addition to the two RuBP carboxylase subunits and the phosphate translocator, the present ATPase seems to be the fourth protein identified in spinach chloroplast envelopes. Due to its special properties, this enzyme might well modulate the exchanges of ions, metabolites and/or proteins between the chloroplast and the cytosol.

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P.-A. Siegenthaler and W. Eichenberger, editors

ARE PHOSPHOLIPID TRANSFER PROTEINS PRESENT IN THE STROMA FROM HIGHER PLANT CHLOROPLASTS ?

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INTRODUCTION

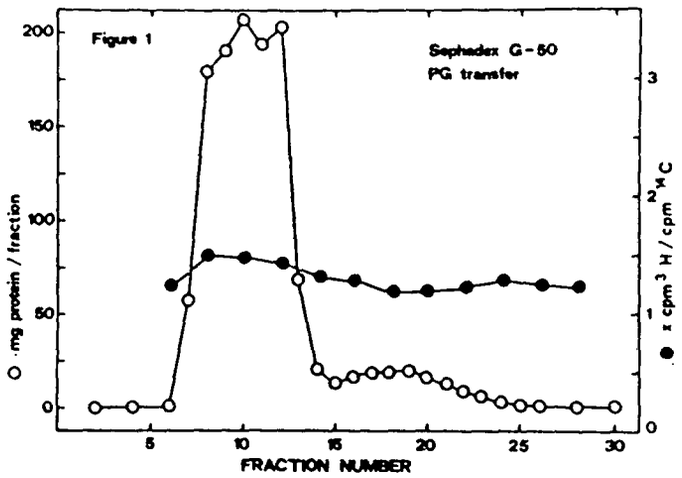
In contrast to most cell membranes and organelles, chloroplasts contain a high proportion of galactolipids and only a small amount of phospholipids, mainly phosphatidylglycerol (PG) and phosphatidylcholine (PC). The ratio PG/PC increases from 0.3 in the outer membrane to 1.3 in the inner membrane of the envelope, whereas ratios between 2 and 4 have been reported for thylakoid membranes (1-2). The outer envelope membrane is thus enriched in PC, which may act as an intermediate molecule involved in the transfer of unsaturated fatty acids (3).

The envelope, but not thylakoid membranes, appears to be involved in the synthesis of fatty acids and lipids (3). Thylakoid lipids should therefore be imported from the envelope either by a mechanism of membrane flow or by a system of lipid carrier. Since a phospholipid transfer protein has been purified from cytosolic extracts of spinach leaf (4), such a protein could also be active in the stroma of chloroplast. The ability of stroma extracts to transfer phospholipids was therefore investigated in the present work, by measuring the transfer of radioactive PG or PC from liposomes to purified potato tuber mitochondria. Mitochondria were chosen to test the lipid transfer activity because they contain much more phospholipids than chloroplast membranes.

MATERIAL AND METHODS

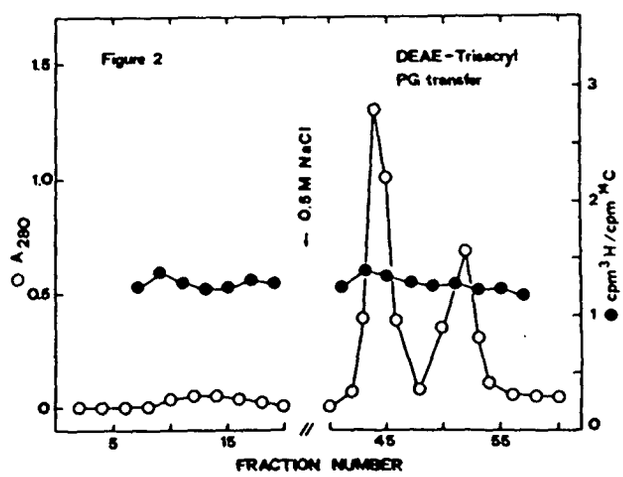
Stroma was prepared from purified intact spinach chloroplasts (5), then dialyzed and concentrated overnight (Mr cutoff : 3500) in a medium containing 50 mM Tris-HCl buffer, pH 7.5, 8 mM mercaptoethanol, 1 mM EDTA and 20% (w/v) polyethyleneglycol 6000. The extract was then loaded on either a gel filtration (Sephadex G-50 or G-75) or an ion exchange (DEAE-Trisacryl or -Sephacel) chromatography column (2.5 x 35 cm) equilibrated in the elution buffer (5 mM Na-phosphate buffer, pH 7.2, 8 mM mercaptoethanol, 3 mM sodium azide). Elution was run at a flow rate of 80 ml/h and 10 ml fractions were collected. Proteins retained on the DEAE column were desorbed with 0.5 M and 1.0 M NaCl added in the elution buffer.

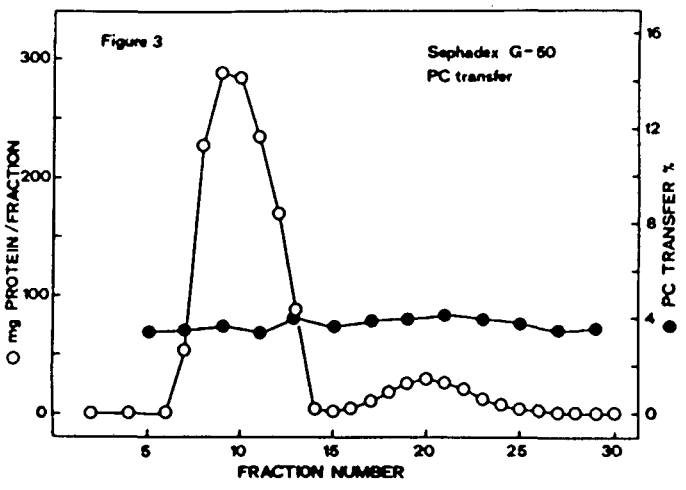
The transfer of radioactive PG or PC from liposomes to purified potato tuber mitochondria was measured as described in (4), except that liposomes were prepared



fractions and was therefore not linked to a specific protein.

When the stroma extract was applied to a DEAE-Trisacryl column (Fig. 2), only a trace amount of unbound protein was found, whereas two major peaks were eluted with 0.5 M NaCl and no protein required higher salt concentrations to be desorbed. No PG transfer activity was associated with either basic or acidic protein fractions. The elution profile of a stroma extract on a DEAE-Sephacel column (Fig. 4) was different from the profile obtained on a DEAE-Trisacryl column : more unbound proteins and an additional peak eluted with 1 M NaCl were found. Only a negligible unspecific PC transfer activity was observed without significant difference between the fractions.

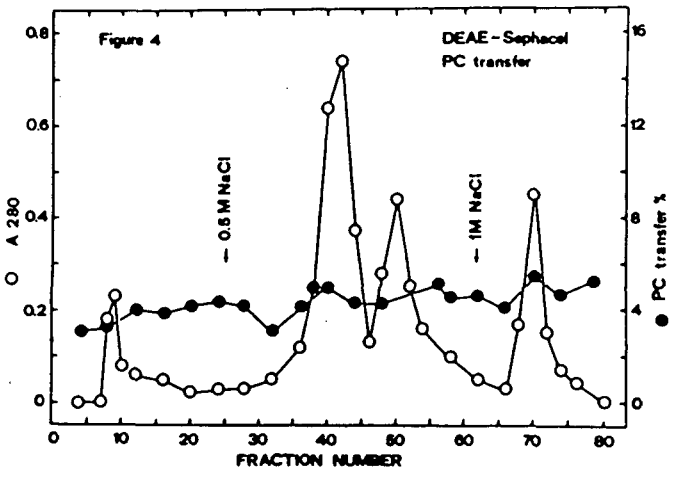




by mixing 300 nmol of either egg yolk PC or PG purified from spinach thylakoids, an aliquot of ^{3}H-phospholipid (250 Bq) and glycerol tri[1-^{14}C] oleate (250 Bq) as a non-transferable tracer.

RESULTS AND DISCUSSION

When the stroma extract was passed through a Sephadex G-50 column (Figs 1 and 3), neither high- nor low-molecular-weight proteins were able to transfer PG from liposomes to mitochondria (Fig. 1). Furthermore, only 3-4% of PC initially present in liposomes was associated with the mitochondrial pellet after a 40 min incubation at 30°C (Fig. 3). However, this background activity was found in all



Alternatively, an ammonium sulfate precipitation of the stroma extract was carried out at 75% saturation. The pellet obtained after centrifugation at 15'000 g for 30 min was dialyzed overnight and loaded on either a Sephadex or a DEAE column. The elution profile of proteins was the same as shown in Figures 1-4 and neither PG nor PC transfer activity were detectable.

The low transfer activity was not increased by the addition of 1 mM $MgCl_2$ nor affected by the pH of the reaction medium over a range from 6 to 8.

In conclusion, the present results indicate a lack of specific PC-transfer proteins and unspecific phospholipid (PG and PC) transfer proteins in the stroma from mature spinach chloroplasts.

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PHYSIOL. PLANT. 63: 187–191. Copenhagen 1985

Calmodulin is not involved in the regulation of exogenous NADH oxidation by plant mitochondria

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The oxidation of exogenous NADH by mitochondria from potato (*Solanum tuberosum* L., cv. Bintje) tubers, measured with different electron acceptors, oxygen, cytochrome *c*, duroquinone and ubiquinone 1, was greatly enhanced under high salt conditions compared to low salt conditions, confirming the stimulatory effect of electrostatic screeening of negative membrane charges by cations. In addition to this non-specific stimulation, the oxidation of exogenous NADH showed a specific dependence on Ca^{2+}. Results presented here suggest that calmodulin was not directly involved in the regulation of exogenous NADH oxidation by potato mitochondria: (1) Calmodulin antagonists were found to inhibit electron flow at several sites in a non-specific manner. (2) Using a phenothiazine-Affi Gel column, it was not possible to demonstrate the presence of calmodulin in Triton X-100 solubilized mitochondria. (3) Fractions eluted from a calmodulin-Sepharose column with EGTA [ethyleneglycol-bis (β-aminoethylether)-N, N, N′, N′-tetraacetic acid] did not display any activity related to mitochondrial electron transport, suggesting that NADH dehydrogenase had no specific affinity for calmodulin. The possible indirect involvement of calmodulin in the regulation of exogenous NADH oxidation by Ca^{2+} is discussed.

Additional key words – Calcium, NADH dehydrogenase, potato tubers.

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Introduction

In contrast to their mammalian counterparts, mitochondria from higher plants and micro-organisms are able to oxidize exogenous NADH at high rates by a dehydrogenase located at the outer surface of the inner membrane and not linked to the rotenone-sensitive energy-coupling site (Hall and Greenawalt 1967, von Jagow and Klingenberg 1970, Douce et al. 1973). In addition to a non-specific stimulation by cations (Møller and Palmer 1981, Møller et al. 1982), the mitochondrial NADH oxidase from several higher plants shows a specific dependence on Ca^{2+} (Møller et al. 1981, Moore and Åkerman 1982). This has been ascribed to a binding of Ca^{2+} to groups essential for NADH oxidation and possibly to a conformational change of the enzyme. However, a Mg^{2+}-dependent system has been described for mitochondria from *Neurospora* (Møller et al. 1982).

Several physiological processes and enzymatic activities in plant cells appear to be regulated by Ca^{2+} and calmodulin: a Ca^{2+} transport ATPase present in the plasma membrane and thus the concentration of free Ca^{2+} in the cytoplasm (Dieter and Marmé 1983), a NAD^+-kinase (Cormier et al. 1981), an ATPase located in the chloroplast envelope (Nguyen and Siegenthaler 1983) and a NAD^+-kinase bound to the outer mitochondrial membrane (Dieter and Marmé 1984). Furthermore, a possible regulatory role of Ca^{2+} and of a calmodulin-type protein in photosynthetic electron transport and photophosphorylation has been suggested (Barr and Crane 1982, Barr et al. 1982). Calmodulin (Hatase et al. 1982) and calmodulin-binding proteins are present

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in the matrix and also bound to the inner membrane of mitochondria from bovine heart (Hatase et al. 1983) and rat liver (Gazzotti et al. 1984). Recent results suggest the presence of calmodulin in the intermembrane space of mitochondria isolated from oat tissues (Biro et al. 1984).

The aim of the present work was first to investigate the Mg^{2+}- or Ca^{2+}-dependence of the exogenous NADH oxidase from potato tubers mitochondria and to localize the site of interaction between Ca^{2+} and the respiratory chain. The second purpose of this work was to investigate the possible involvement of calmodulin in the regulation of the oxidation of exogenous NADH by potato tuber mitochondria.

Abbreviations – BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid, sodium salt; EGTA, ethyleneglycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone; MOPS, 3-(N-morpholino)propane sulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

Materials and methods

Isolation of mitochondria

Potato tubers (*Solanum tuberosum* L., cv. Bintje) were obtained from a local market and stored in the dark at 4°C. Tubers were peeled, cut in small cubes and washed in distilled water. Batches of 500 g were homogenized in a 4.5 l Waring Blendor for 15 s (low position) in 500 ml of the extraction medium containing 0.4 *M* sucrose, 50 m*M* K^+-MOPS,pH 7.8, 5 m*M* EDTA, 5 m*M* cysteine-HCl, 10 m*M* mercaptoethanol and 0.1% (w/v) BSA. The homogenate was filtered through two layers of gauze and two layers of Miracloth. The filtrate was centrifuged at 1500 *g* for 15 min, then at 12000 *g* for 15 min. The crude mitochondrial pellet was resuspended in a minimal volume of the washing medium containing 0.4 *M* sucrose, 10 m*M* K^+-MOPS, pH 7.2 and 0.1% (w/v) BSA and layered on the top of a discontinuous gradient made of four 7.5 ml layers of sucrose (2.25, 2.10, 1.55 and 1.05 *M*). All sucrose solutions contained 10 m*M* K^+-phosphate, pH 7.2 and 1 m*M* EDTA. Centrifugation was carried out in an ultracentrifuge Beckman L8-55 at 90000 *g* (max) for 60 min (rotor SW-27). After centrifugation, mitochondria were collected from the 1.55–1.05 *M* sucrose interface with a Pasteur pipette and slowly diluted in 5 volumes of the washing medium. After centrifugation at 12000 *g* for 15 min, the purified mitochondria were suspended in a minimal volume of the washing medium. As estimated by measuring the succinate-cytochrome c oxido-reductase in isotonic and hypotonic medium, the apparent integrity of the outer mitochondrial membrane varied between 80 and 90%.

Solubilization of mitochondria

Mitochondria were solubilized by the addition of 20 mg of Triton X-100 (Merck, Darmstadt, FRG) per 10 mg of protein and the suspension was stirred for 10 min at 4°C. Non-solubilized membrane proteins were removed by centrifuging at 20000 *g* for 10 min and discarding the pellet.

Affinity chromatography

After the addition of 1 m*M* $CaCl_2$, the supernatant containing the solubilized proteins was loaded on either a 20 ml phenothiazine-Affi Gel (Biorad Laboratories, Richmond, CA, USA) or a 25 ml calmodulin-Sepharose affinity column (Dieter and Marmé 1981). After loading, the column was washed with 200 ml of a medium containing 0.5 *M* NaCl, 0.3 *M* sucrose, 50 m*M* Tris-HCl, pH 7.8, 0.05% (w/v) Triton X-100, 1 m*M* mercaptoethanol and 1 m*M* $CaCl_2$. Proteins retained on either column were eluted with the same buffer without $CaCl_2$ but with 5 m*M* EGTA.

Activity measurements

The electron flow from NADH to oxygen, duroquinone or ubiquinone-1 was followed by measuring the decrease in absorbance at 340 nm, using 374 nm as the reference wavelength in an Aminco DW-2a dual wavelength spectrophotometer ($\varepsilon_{340\text{-}374}$ = 3.73 mM^{-1} cm^{-1}). These activities were measured either in a low cation medium [0.4 *M* sucrose, 10 m*M* K^+-phosphate, pH 7.2, 0.1% (w/v) BSA] or a high cation medium [0.25 *M* sucrose, 100 m*M* KCl, 10 m*M* K^+-phosphate, pH 7.2, 0.1% (w/v) BSA], with 0.25 m*M* NADH, 0.3–0.6 mg of mitochondrial protein and either 0.5 μ*M* FCCP, 1 m*M* KCN plus 1 m*M* duroquinone (Sigma Chemical Co., St. Louis, MO, USA) or 1 m*M* KCN plus 2 m*M* ubiquinone-1 (Hoffmann-La Roche, Basel, Switzerland). The electron flow from either NADH or succinate to cytochrome *c* was measured in a medium containing 5 m*M* K^+-phosphate, pH 7.2 (with or without 100 m*M* KCl), 1 m*M* KCN, 80 μ*M* oxidized cytochrome *c*, 1 m*M* NADH or 10 m*M* succinate using 0.2–0.4 or 0.4–0.6 mg of mitochondrial protein. The reduction of cytochrome *c* was followed by measuring the absorbance increase at 550–540 nm ($\varepsilon_{550\text{-}540}$ = 19 mM^{-1} cm^{-1}). The cytochrome oxidase (EC 1.9.3.1) activity was measured at 550–540 nm in a medium containing 10 m*M* K^+-phosphate buffer, pH 7.0 (plus or minus 100 m*M* KCl), 50 μ*M* reduced cytochrome *c* and 0.2–0.4 mg of mitochondrial protein. All measurements were carried out at 25°C. When required, mitochondria were preincubated for 1 min in the presence of 1 m*M* $CaCl_2$, 1 m*M* EDTA, 1 m*M* EGTA, 0.1 m*M* phenothiazine (Sigma), 0.1 m*M* chlorpromazine (Sigma) or 30 μg ml^{-1} of compound 48/80 (Sigma) before the addition of the substrate. The activity of c-AMP phosphodiesterase (EC 3.1.4.17) was

Tab. 1. Effect of cations, calcium and EGTA on the activity of different oxido-reductases. With NADH as an electron donor, activities are expressed as nmol NADH oxidized (mg protein)$^{-1}$ min^{-1}. Other activities are presented as nmol cytochrome c reduced or oxidized (mg protein)$^{-1}$ min^{-1}. Mean (± SE) of 3–4 independent experiments.

Oxido-reductase assayed	Low cation medium	High cation medium		
		Control	+ 1 m*M* $CaCl_2$	+ 1 m*M* EGTA
NADH-oxygen	95±2	522±23	623±57	5±2
NADH-cytochrome *c*	114±8	204±9	203±2	95±13
NADH-duroquinone	114±17	470±32	666±7	24±8
NADH-ubiquinone 1	379±17	1080±131	1580±73	606±77
Succinate-cytochrome *c*	97±9	76±6	66±8	88±9
Cytochrome c-oxygen	105±8	239±29	225±5	244±24

measured according to Dieter and Marmé (1980). Protein was determined according to Lowry et al. (1951). Calmodulin was isolated from bovine brain according to Caldwell and Haug (1981).

Results and discussion

All activities tested, except the succinate-cytochrome *c* oxido-reductase, were much higher in a high than in a low cation medium (Tab. 1). These results confirm that electrostatic screening of fixed negative charges by cations stimulates not only the oxidation of exogenous NADH but also other rate-limiting steps in mitochondrial electron transport (Møller and Palmer 1981, Møller et al. 1984). In addition, only Ca^{2+} (Tab. 1), but not Mg^{2+} (results not shown), further stimulated the electron flow from NADH to oxygen, duroquinone or ubiquinone-1 in a high cation medium. All reactions involving NADH as an electron donor were strongly inhibited by chelators like EGTA (Tab. 1) or EDTA (results not shown). Furthermore, only Ca^{2+}, but not Mg^{2+}, was able to release the inhibition caused by either EDTA or EGTA. Table 1 shows that a Ca^{2+}-dependent NADH oxidase was present in potato tuber mitochondria, like in Jerusalem artichoke tubers (Møller et al. 1981). However, no Mg^{2+}-dependent system, like in *Neurospora* (Møller et al. 1982) could be detected. Ca^{2+} might interact with the dehydrogenase itself, at or before the site of reduction of quinones, as previously suggested by Cowley and Palmer (1978) and by Møller et al. (1983).

Tab. 2. Effect of calmodulin antagonists on the activity of different oxido-reductases. Values were determined under high cation conditions and expressed as % inhibition (−) or stimulation (+) as compared with control activities (see Tab.1).

Oxido-reductase assayed	Phenothiazine (0.1 m*M*)	Chlorpromazine (0.1 m*M*)	Compound 48/80 (30 μg ml^{-1})
NADH-oxygen	−33	−35	−45
NADH-cytochrome *c*	−30	−39	−67
NADH-duroquinone	−24	No effect	+46
NADH-ubiquinone 1	−15	No effect	+59
Succinate-cytochrome *c*	−40	−48	−62
Cytochrome *c* – oxygen	−23	−29	−64

Tab. 3. Recovery of oxido-reductase activities after affinity chromatography of Triton X-100 solubilized mitochondria. Activities are expressed as μmol NADH oxidized min^{-1}. The activity of fractions eluted with EGTA was measured in the presence of an exeess of $CaCl_2$.

Fraction	NADH-ubiquinone oxido-reductase	
	Total activity	EGTA-sensitive activity
A. Phenothiazine-Affi Gel column		
Loaded on the column	0.57	0.19
Eluted with 1 m*M* $CaCl_2$	0.42	0.10
Eluted with 5 m*M* EGTA	0	0
B. Calmodulin-Sepharose column		
Loaded on the column	1.40	0.63
Eluted with 1 m*M* $CaCl_2$	1.28	0.41
Eluted with 5 m*M* EGTA	0	0

Electron flow from NADH to oxygen or to exogenous cytochrome *c* was inhibited by relatively low concentrations of calmodulin antagonists, like phenothiazine, chlorpromazine or compound 48/80 (Tab. 2). Although phenothiazine inhibited slightly and chlorpromazine did not affect NADH-duroquinone and NADH-ubiquinone-1 oxido-reductases, compound 48/80 strongly stimulated both reactions. Furthermore, while cytochrome *c* oxidase and succinate-cytochrome *c* oxido-reductase activities were insensitive to either Ca^{2+} or EGTA (Tab. 1), both reactions were inhibited by calmodulin antagonists (Tab. 2). Finally, the addition of 30 μg calmodulin to the reaction medium in the presence of 10 μ*M* or 1 m*M* $CaCl_2$ did not further stimulate the NADH-cytochrome *c* oxido-reductase activity (results not shown). These results indicate that calmodulin antagonists acted as unspecific inhibitors at several sites on the respiratory chain and therefore question their use to identify membrane-bound calmodulin-dependent

processes. Indeed, calmodulin antagonists might bind to mitochondrial inner membranes by non-selective hydrophobic interactions and act as unspecific inhibitors of the respiratory chain. In mitochondria from different sources, phenothiazine drugs inhibit several calmodulin-independent activities, like the ATPase (Ruben and Rasmussen 1981), the electron transport at the level of cytochrome oxidase and cytochrome b-c_1 (Chazotte and Vanderkooi 1981, Cheah and Waring 1983, Dunn et al. 1984) and Ca^{2+} translocation across the inner mitochondrial membrane (Vale et al. 1983).

It is however conceivable that calmodulin could act as a Ca^{2+}-binding, regulatory subunit of the NADH dehydrogenase or that the enzyme might show an affinity for calmodulin. Proteins solubilized from intact mitochondria were therefore loaded in the presence of Ca^{2+} onto either a phenothiazine-Affi Gel or a calmodulin-Sepharose affinity column, and eluted with EGTA. Neither the NADH-ubiquinone 1 oxido-reductase activity (Tab. 3) nor any significant amount of protein (results not shown) were retained on a phenothiazine affinity column. Moreover, the EGTA-eluted fractions from the phenothiazine-Affi Gel column did not stimulate the calmodulin-dependent c-AMP phosphodiesterase activity measured in vitro (results not shown). These negative results suggest that highly intact potato tuber mitochondria contain no significant amount of calmodulin, in contrast to mitochondria from oat tissues, which appear to contain calmodulin in their intermembrane space (Biro et al. 1984). Although some protein was retained on a calmodulin-Sepharose affinity column and eluted with EGTA (results not shown), the NADH-duroquinone oxido-reductase activity was eluted mostly with 1 mM $CaCl_2$ (Tab. 3). The unbound enzyme, released by $CaCl_2$ only, may be due to the different NADH dehydrogenases encountered in plant mitochondria. Their activity was partially inhibited by EGTA. These results suggest that NADH dehydrogenases had no specific affinity for calmodulin.

In conclusion, our results do not support a direct involvement of calmodulin in the regulation of exogenous NADH oxidation by potato tuber mitochondria. However, since calmodulin is involved in the regulation of the concentration of free Ca^{2+} in the cytoplasm, calmodulin could indirectly affect the Ca^{2+} concentration in the intermembrane space of mitochondria and hence the activity of the NADH dehydrogenase located at the outer surface of the inner membrane.

Acknowledgements – The technical assistance of B. Bekele is gratefully acknowledged. This work was supported by the Swiss National Science Foundation (Grant 3.417.0.83 to P.A.S.) and in part by the Emil-Barrel Foundation. Ubiquinone-1 was kindly supplied by Hoffmann-La Roche (Basel, Switzerland).

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Purification and some properties of an Mg^{2+}-, Ca^{2+}- and calmodulin-stimulated ATPase from spinach chloroplast envelope membranes

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Spinach chloroplasts display an ATPase activity which is associated with the envelope. This envelope-bound activity is stimulated by Ca^{2+}, Mg^{2+} and calmodulin (Nguyen, T.D. and Siegenthaler, P.A. (1983) FEBS Lett. 164, 67–70). The Triton X-100-solubilized enzyme was retained specifically on a calmodulin-Sepharose affinity column in the presence of calcium. The fractions eluted by EGTA contained two proteins characterized by p*I* values of 7.3 and 6.0 (isoelectric focusing). Both proteins, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-polyacrylamide gel electrophoresis), were resolved into a single polypeptide having an identical apparent M_r of 65 000. This suggests that the two initial proteins might be isoelectric variants. However, the amount of the enzyme fraction obtained by the calmodulin-Sepharose column was small and the ATPase activity was very labile. A linear glycerol gradient allowed the recovery of a greater amount of the enzyme which was, however, only partially purified, but the activity of which was much more stable. Electrophoresis of the ATPase-containing fractions in a native polyacrylamide gradient gel permitted the separation of a 260 kDa protein which was resolved by SDS-polyacrylamide gel electrophoresis into a single polypeptide of 65 kDa. Thus, the chloroplast envelope-bound ATPase might be a tetramer (260 kDa) consisting of 4 identical monomers (65 kDa). The purified ATPase had properties similar to that of the envelope-bound enzyme. The K_m value for ATP was 0.45 mM. The activity was stimulated by Ca^{2+} and Mg^{2+}, and further enhanced by calmodulin. The physiological significance of the chloroplast envelope-bound ATPase is discussed.

Introduction

Chloroplast envelopes contain at least 21 proteins as separated by isoelectric focusing [1] and at least 70 polypeptides, 40 of which are integral membrane components characterized by their high M_r ($M_r > 50\,000$) and their hydrophobicity [2]. Recently, procedures to separate fractions enriched in outer and inner envelope membranes from pea and spinach chloroplasts have been developed [3,4]. Accordingly, it has been possible to assign specific polypeptides and enzymatic activities to each of these two fractions [5,6]. For instance, the phosphate translocator as well as several enzymes involved in the synthesis of galactolipids and phosphatidylglycerol are associated with the fraction enriched in inner envelope membranes [3,5,7–9]. However, there is still controversy about the distribution of some of these enzymes between the two envelope membranes. In addition, the identity of the proteins and poly-

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

peptides found in chloroplast envelopes is unknown with the exception of the large and small subunits of ribulose-1,5-bisphosphate carboxylase, both of which are probably contaminants, and of the phosphate translocator.

Of special interest is the presence of an Mg^{2+}-dependent ATPase in the fraction enriched in inner envelope membranes [5,10]. This envelope-bound enzyme, first described in 1973 [11], is Mg^{2+}- and/or Mn^{2+}-dependent [12] and insensitive to *N,N'*-dicyclohexylcarbodiimide. Recently, the chloroplast envelope-bound ATPase activity has been shown to be stimulated by 0.15 mM Ca^{2+} and by 5 mM Mg^{2+}, and to be further enhanced by calmodulin. The enzyme is especially sensitive to Na_3VO_4, $LaCl_3$ and oligomycin [13]. However, the M_r and the oligomeric structure of this ATPase is still unknown.

In plant tissues, calmodulin modulates the activity of at least three enzymes: NAD^+-kinase, Ca^{2+}-ATPase and quinate/NAD^+ oxidoreductase [14]. Calmodulin is found in spinach [15], pea [16] and wheat [17] leaves and is present mainly in the cytosol (90%) and to a lesser extent in mitochondria (5–9%), chloroplasts (1–2%) and the microsomal fraction (less than 1%) [17]. In chloroplasts, calmodulin appears to be confined in the stroma [16]. Calmodulin antagonists such as chlorpromazine or phenothiazine inhibit electron transport in photosystem II of spinach thylakoids [18] and the proton gradients associated with photophosphorylation [19].

Thus, we postulated that, among the 21 chloroplast-envelope proteins separated by isoelectric focusing [1], the envelope-bound ATPase might display a specific affinity for calmodulin and that its activity might well be modulated by this protein [13]. The aim of this investigation was to test this hypothesis by attempting to retain specifically the envelope-bound ATPase on a calmodulin-Sepharose affinity column in the presence of calcium and to release it with EGTA. This attempt was successful and allowed the investigation of some biochemical properties of the purified enzyme. Thus, the ATPase is, to our knowledge, the second protein, after the phosphate translocator, to be characterized in the chloroplast envelope.

Materials and Methods

Chemicals. All reagents were of the highest purity and purchased from Fluka, except for Triton X-100 which was obtained from Merck, SDS from Serva, $CaCl_2$, EGTA, $Na_2 \cdot ATP$ from Sigma, CNBr-Sepharose 4B and molecular weight protein kits from Pharmacia.

Preparation of envelopes. Envelope membranes were prepared as described by Nguyen and Siegenthaler [13]. Usually, 3 kg of deveined spinach leaves were homogenized in 6 l of extraction medium (25 mM Tricine-NaOH (pH 7.8), 300 mM sucrose, 0.1% defatted bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride (PMSF)). The homogenate was filtered through eight layers of cheesecloth and centrifuged at $1500 \times g$ for 5 min. The pellets of crude chloroplasts were resuspended in the extraction medium and centrifuged at $2000 \times g$ for 5 min through a 40% Percoll layer having the same composition as the grinding medium. The pellets of intact chloroplasts were washed in the extraction medium and spun down at $2000 \times g$ for 10 min. Intact purified chloroplasts were lysed by osmotic shock in 10 mM Tricine-NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM PMSF (medium A) and thylakoids were partially removed by centrifugation at $12000 \times g$ for 10 min. The supernatant was layered on the top of a two-step sucrose gradient: 1.0 M (10 ml) and 0.6 M (10 ml) in medium A and centrifuged at $95000 \times g$ (R_{max}) for 1 h. The envelope fraction banded at the sucrose interface. The envelopes were collected, diluted 4–5-fold with 10 mM Tricine-NaOH (pH 7.8) and sedimented at $122000 \times g$ (R_{max}) for 45 min.

ATPase assays. ATPase activity was routinely determined by the method described earlier [13] in a reaction mixture (250 µl) containing 50 mM Tris-HCl (pH 7.8), 300 mM sucrose, 10–15 µg envelope protein or 2 µg purified enzyme (15 min at 37°C). The coloration of the phosphomolybdate complex was stabilized as described by Penttinin [21]. Concentrations of ATP, $MgCl_2$ and $CaCl_2$ are indicated in the legends of the figures. Free (ionized) calcium and magnesium concentrations were controlled in the submicromolar range by utilizing the buffering ligand EGTA as described by Pershadsingh and McDonald [23] and calculated according to Schatzmann [24].

Electrophoretic methods. Conditions for isoelectric focusing and SDS-polyacrylamide gel electrophoresis were as in Ref. 1. The native gel system was performed in absence of detergent as described by Davis [22] in a linear gradient of acrylamide (5–30%) at 150 V for 16.7 h (2500 V · h).

The molecular weights (M_r) of native proteins were estimated by plotting the log M_r of standard proteins (high molecular weight kit of Pharmacia) versus their relative distance of migration. A deviation of about 15% for the higher M_r values has been observed and was corrected for by using purified ribulose-1,5-bisphosphate carboxylase (isolated according to Joyard et al. [21]) as a reference protein [25].

Calmodulin-Sepharose affinity chromatography. Envelope membranes were resuspended in buffer A (50 mM Tris-HCl (pH 7.8), 0.3 M sucrose, 20% glycerol, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 1 mM 2-mercaptoethanol), solubilized in Triton X-100 (1 mg Triton/mg protein) for 10 min at 4°C and centrifuged at 150 000 × *g* for 1 h. The supernatant was then applied on a calmodulin-Sepharose 4B column (1.5 × 15 cm) which was equilibrated in buffer B (buffer A + 0.05% Triton X-100). Transmittance at 280 nm was recorded by a LKB Uvicord. The column was washed first with buffer B, then with buffer B + 0.5 M NaCl and finally with buffer B. Proteins were eluted with buffer C (buffer B in which $CaCl_2$ is replaced by 1 mM EGTA). All assays were made immediately after elution to avoid the loss of enzyme activity as much as possible.

Linear gradient of glycerol. Envelope membranes were resuspended in 50 mM Tris-HCl (pH 7.8) containing 1 mM 2-mercaptoethanol, solubilized in Triton X-100 (2 mg Triton X-100/mg protein) for 10 min at 4°C and centrifuged at 150 000 × *g* for 1 h. The supernatant was collected and glycerol was added to a concentration of 20% (v/v). The mixture (about 2 mg protein) was layered on top of a linear gradient of glycerol (25–40% (v/v) in 50 mM Tris-HCl (pH 7.8), 1 mM 2-mercaptoethanol, 0.05% Triton X-100). After a centrifugation at 140 000 × *g* for 14 h, fractions were collected and assayed for protein and ATPase activity. Relative glycerol concentrations were estimated from the refraction index.

Other methods. Protein was determined as in Ref. 26, using bovine serum albumin as reference. Calmodulin was purified from bovine brain [27]. The coupling of calmodulin to Sepharose 4B was carried out essentially according to Pharmacia instructions.

Results and Discussion

Purification of the chloroplast envelope-bound ATPase

Since calmodulin was found to enhance the activity of the envelope-bound ATPase [13], we postulated that the enzyme might have a specific affinity for calmodulin. We attempted, therefore, to retain specifically the ATPase on a calmodulin-Sepharose affinity column in the presence of calcium, as shown in Fig. 1. Most of envelope proteins were eluted by a buffer containing $CaCl_2$. After two washings with a mixture of NaCl + $CaCl_2$, and with $CaCl_2$ alone to remove proteins which were adsorbed on the column in a non-specific manner, an EGTA-washing allowed the elution of most of the ATPase (fraction 31–37). These fractions contained only about 1% of the total envelope proteins loaded on the column and about 4% of the total ATPase activity which was found after solubilization of chloroplast envelopes by Triton X-100. The purification factor varied between 90 and 100. Protein fractions were then concentrated and separated by isoelectric focusing.

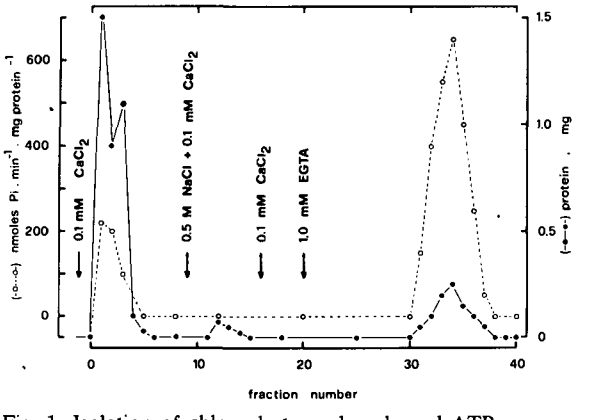

Fig. 1. Isolation of chloroplast envelope-bound ATPase on a calmodulin-Sepharose affinity column. ●, protein; ○, ATPase activity, tested in the presence of 4 mM ATP and 4 mM $MgCl_2$.

Fig. 2 shows that the EGTA-eluted fractions contained two proteins, characterized by p*I* values 7.3 and 6.0. It is noteworthy that, among the 21 proteins of chloroplast envelope separated by isoelectric focusing (Fig. 2A and Ref. 1), only two of them presented a specific affinity for calmodulin. To determine the subunit composition of the two proteins obtained by isoelectric focusing (first dimension) we subjected them to an electrophoretic separation in a polyacrylamide gel containing SDS (second dimension). Fig. 3 shows that both proteins were resolved into polypeptides having an identical apparent M_r of 65000. This band was visible in the gel containing the total polypeptides of chloroplast envelopes (Fig. 3E) and displays a well-separated peak in a densitometric tracing of the gel [1]. The presence of two proteins, characterized by different native charges, but giving rise to only one type of subunit may be due to isoelectric variants, as was also observed for ribulose-1,5-bisphosphate carboxylase [28,29]. In addition, it is surprising that none of the four envelope polypeptides (50, 33, 27 and 17 kDa) which have been found to be 'bound' by radioactive calmodulin [30] are retained on the calmodulin-Sepharose column.

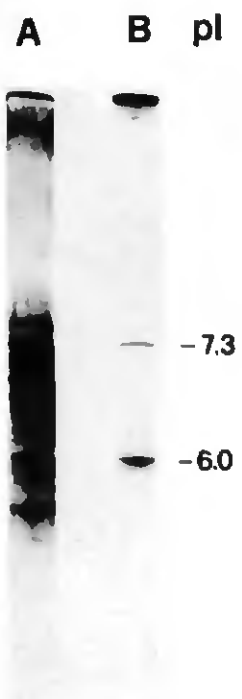

Fig. 2. Separation of proteins by isoelectric focusing. A, chloroplast envelope protein; B, EGTA-fraction eluted from the calmodulin-Sepharose column. Figures correspond to p*I* values.

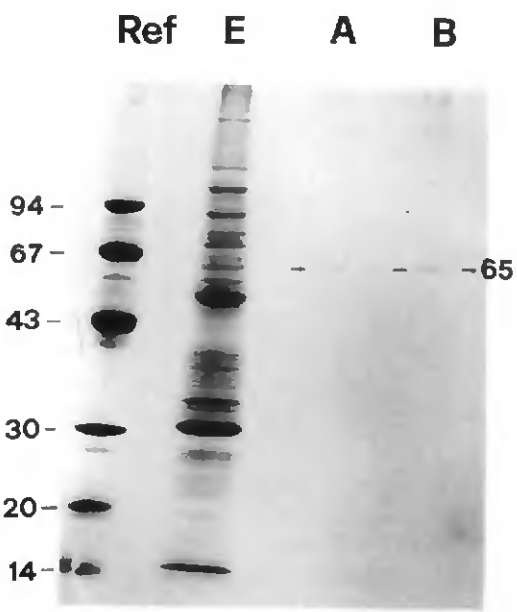

Fig. 3. Electrophoretic separations in SDS-polyacrylamide gel (second dimension) of proteins obtained by isoelectric focusing (first dimension) in Fig. 2B. Ref, standard polypeptides (kDa); E, control envelope polypeptides; A, protein having a p*I* of 6; B, protein having a p*I* of 7.3.

Next, it was desirable to estimate the apparent M_r of the protein corresponding to the ATPase activity. Although the calmodulin-Sepharose affinity column allowed the separation of the ATPase from the other proteins of chloroplast envelopes, this method presented three drawbacks: (1) the two salt washings of the calmodulin-Sepharose column, which were found to be necessary to ensure a good purification of the enzyme, partially inactivated the ATPase; (2) the EGTA-eluted fractions diluted too much the enzyme which was further inactivated during the subsequent concentrating procedure with poly(ethylene glycol); (3) the amount of enzyme collected by this column method was too low to undertake systematic electrophoretic studies of the enzyme. Therefore, we investigated other means to purify and isolate the ATPase. In experiments intended to improve the solubilization of chloroplast envelope membrane,

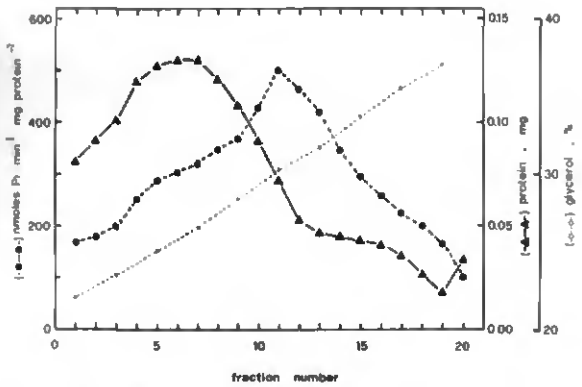

Fig. 4. Separation of chloroplast envelope proteins, Triton X-100-solubilized, in a linear glycerol gradient (20–40%). ▲, proteins; ●, ATPase activity, tested in the presence of 4 mM ATP and 4 mM $MgCl_2$.

we found that an incubation at 4°C for 10 min in 0.3 M sucrose, 50 mM Tris-HCl (pH 7.8), 0.3 mM Triton X-100 (per 40 μg envelope protein/ml) in the presence of 20% (v/v) glycerol were the best conditions to solubilize the envelope (68% of total protein) and, simultaneously, to preserve the ATPase activity (25% inactivation after 6 days at 4°). These results prompted us to attempt the separation of the enzyme in a linear glycerol gradient. Fig. 4 shows the distribution of chloroplast envelope proteins solubilized in Triton X-100 and the ATPase activity in the different fractions. Most of the proteins migrated slowly and remained at the top of the glycerol gradient, whereas proteins associated with the ATPase activity were displaced towards the middle of the gradient. Fractions 8–15 represented 38% of the total protein loaded on the gradient and 50% of the total ATPase activity which was found after solubilization of chloroplast envelopes by Triton X-100. Furthermore, they displayed an activity of 500 nmol P_i/min per mg protein which was 3-times higher than that of the envelope-bound enzyme. The activity of this partially purified enzyme remained unchanged for at least 48 h at 0°C. The collected fractions were then concentrated and loaded on a native polyacryl-

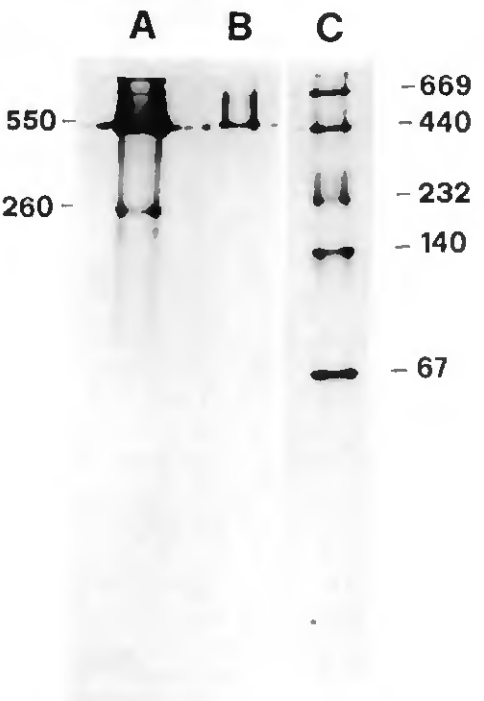

Fig. 5. Electrophoretic separation of proteins in a native linear polyacrylamide gradient (5–30%). A, proteins of fractions 8–15 (see Fig. 4); B, ribulose-1,5-bisphosphate carboxylase, purified according to Ref. 2; C, standard proteins of high molecular weight kit, in kDa (Pharmacia).

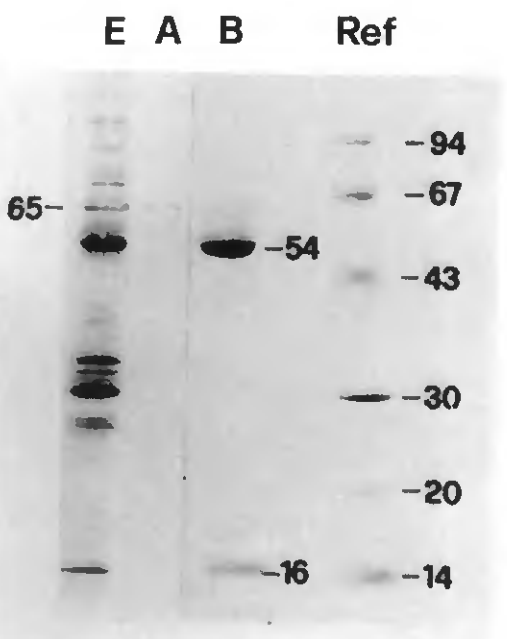

Fig. 6. Electrophoretic separation in SDS-polyacrylamide gel (2nd dimension) of proteins separated on the native gel (1st dimension, Fig. 5). E, control envelope polypeptides; A, 260 kDa protein; B, 550 kDa protein; Ref, standard proteins in kDa.

amide gradient gel. Fig. 5A shows two main bands in the gel. The first, larger band had exactly the same mobility as the purified carboxylase (compare Fig. 5A and B). The second, smaller band corresponded to a protein having an apparent M_r of 260000 (corrected values, see Materials and Methods). Each of these bands were subjected to a second electrophoresis in a polyacrylamide gel containing SDS. Fig. 6A shows that the 260 kDa protein was resolved into a single polypeptide of 65 kDa. In contrast, the 550 kDa protein was resolved into two polypeptides, 54 and 16 kDa, corresponding to the large and small subsunits of the ribulose-1,5-bisphosphate carboxylase (Fig. 6B). Although the glycerol gradient did not allow a complete purification of the enzyme, it enabled us to get enough material for two subsequent electrophoreses (native gel in the first dimension followed by a SDS gel in the second dimension). In conclusion, the chloroplast envelope protein, which is associated with the ATPase activity, might be a tetramer of 260 kDa, consisting of four identical monomers of 65 kDa. However, since no detergent was present in the native gel, one cannot rule out completely the formation of aggregates between the 65 kDa polypeptides, giving rise to the 260 kDa protein observed in Fig. 5A.

The chloroplast envelope ATPase enzyme bears no resemblance to the ATPase from chloroplasts [31,32], mammalian mitochondria [33] and bacteria [33,34]. For instance, the M_r of the CF_1-ATPase from both spinach thylakoids [31] and *Chlamydomonas* [32] is about 40000 and that from bovine heart mitochondria [33], *Escherichia coli* [34] and the thermophilic bacterium PS3 [33] is about 38000. In addition, the composition of these latter enzymes is consistent with an $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit stoichiometry, which emphasizes the greater complexity of these ATPases compared to that of the chloroplast envelope. By contrast, this latter enzyme is similar to ATPases from several other cell types (see Refs. 35–37 and Refs. therein) in that, upon SDS-polyacrylamide gel electrophoresis, they display a single polypeptide band of about 100000 (65000 for the envelope ATPase, see Figs. 3 and 6). Thus, the M_r and the subunit composition of the chloroplast envelope-bound ATPase appear to be quite distinct from other known ATPases.

Some properties of the purified chloroplast envelope ATPase

To test the properties of the ATPase, we used the EGTA-eluted fractions of the calmodulin-Sepharose column, because the enzyme was much more pure than that obtained on a glycerol gradient. However, the success of these experiments requires that the enzyme be used immediately after the collection of the fractions. The activity of the purified ATPase as a function of ATP concentration followed Michaelis-Menten kinetics with an apparent K_m value for ATP of 0.45 mM as determined by the double reciprocal plot of $1/V$ vs. $1/S$ (results not shown). Compared to the value reported for the bound-envelope ATPase [12,13] the purified enzyme had a greater affinity for ATP. However, it was still lower than that of the chloroplast envelope-bound ATPase from *Pisum sativum* [10]. It was also found that above 4 mM of ATP the ATPase activity was progressively inhibited. This was due to a lack of $MgCl_2$ in the reaction mixture, since an ATP/Mg^{2+} molar ratio of 1 was necessary to obtain maximal activity. When sufficient Mg^{2+} was added to obtain a 1 : 1 ratio with ATP at 10 mM, the activity increased to its maximum.

The ATPase had a basal activity, varying between 80 and 120 nmol/mg protein per min, which was independent of the presence of divalent cations (see control in Fig. 7). However, the ATPase activity was stimulated by divalent cations with

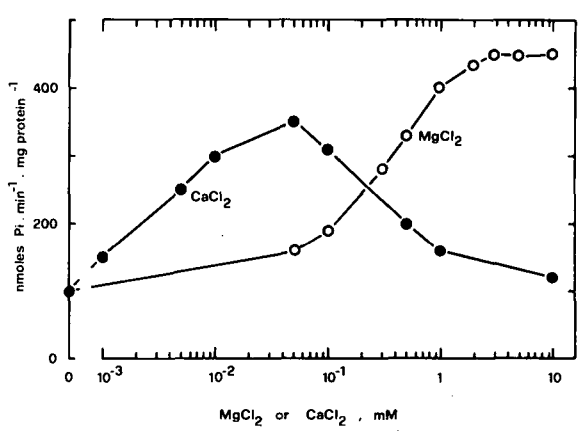

Fig. 7. Stimulation of ATPase activity by $MgCl_2$ and $CaCl_2$. The enzyme was purified as shown in Fig. 1. The reaction mixture contained 4 mM ATP and salts at the concentrations indicated.

maximal rates at 0.05 mM $CaCl_2$ and 3–10 mM $MgCl_2$ (Fig. 7). It is noteworthy that above 0.05 mM, $CaCl_2$ did not stimulate the enzyme activity but inhibited it. The behaviour of the purified ATPase towards cations, namely toward $CaCl_2$ and $MgCl_2$, was very similar to that of the envelope-bound enzyme [13]. However, maximal activity was reached at lower concentrations: 0.05 instead of 0.15 mM $CaCl_2$ and 3 instead of 5 mM $MgCl_2$. It is noteworthy that the nature of anions accompanying the Mg^{2+} cation was not found to be critical [13], although $MgCl_2$ induced a greater activity of the envelope-bound ATPase (195 nmol P_i/mg protein/min) than $Mg(NO_3)_2$ (113 nmol) or $MgSO_4$ (108 nmol). However, the presence of Cl^- in the incubation medium might explain, at least in part, the basal ATPase activity observed in both the envelope-bound [13] and purified enzyme (Fig. 7).

The observation that the ATPase was the only chloroplast envelope protein to be retained on a calmodulin-Sepharose column (Fig. 1) suggested that the enzyme had a specific affinity for calmodulin and, therefore, could be activated by or dependent on calmodulin. Both the envelope-bound ATPase and purified enzyme were stimulated by calmodulin (results not shown). The stimulation was greater for the purified ATPase (45%) than for the bound enzyme (30%). It is the first time that a spinach chloroplast envelope-bound enzyme has been found to be stimulated by calmodulin. Recently, a Ca^{2+}, calmodulin-dependent NAD^+ kinase activity was reported to be localized in pea [38], but not in spinach chloroplast envelopes [39].

The chloroplast envelope-bound ATPase appears to be modulated by Ca^{2+}, Mg^{2+} and calmodulin [13]. Similar properties have now been found for the purified enzyme. Thus, one can expect that, in the dark, the ATPase activity, which is associated with the inner membrane of the envelope [5,10], is very low. Indeed, no free Ca^{2+} is present in the stroma [40], and even the dark level of ATP (approx. 0.6 mM) which represents 30–60% of the maximum ATP level found in saturating light [41] is not sufficient to sustain high ATPase activity. Furthermore, based on the assumption that the concentration of free Mg^{2+} in the stroma is about 10 mM [40], one can calculate that the ATP/Mg^{2+} molar ratio is about 0.06. Taking into consideration that $Mg\,ATP^{2-}$ is the true substrate of the enzyme [13] and that an excess of Mg^{2+} inhibits the enzyme activity, the ATPase should be almost completely inactivated under dark conditions. Upon illumination, one can postulate two activation phases of the enzyme activity. First, Ca^{2+} is taken up into chloroplasts [42]. In the presence of calmodulin [16] and increasing amounts of ATP, due to photophosphorylation, the ATPase is stimulated. In a second phase, when higher concentrations of Ca^{2+} inhibit the enzyme, the increase in pH [40], ATP [41] and Mg^{2+} [40] concentrations should provide favorable conditions for a further stimulation of the ATPase. Since energy, in the form of ATP, is required to move polypeptides across the chloroplast envelope [43], it is tempting to associate the chloroplast envelope-bound ATPase with the transport of cytoplasmically synthesized polypeptides into chloroplasts. However, this hypothesis does not exclude the possibility that an active mechanism, mediated by the chloroplast envelope-bound ATPase, might be involved in H^+ efflux and K^+ uptake, as postulated by Maury et al. [44], or in the light-induced Ca^{2+} uptake by intact chloroplasts. Thus, Ca^{2+} seems to be involved not only in the regulation of stroma enzymes, such as NAD^+ kinase [45] and fructose bisphosphatase [46], and in the water-splitting system in thylakoids [47,48], but also in the control of polypeptide and ion import through chloroplast envelope.

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