The extraction from maize (*Zea mays*) root cells of membrane-bound protein with Ca^{2+} -dependent ATPase activity and its possible role in membrane fusion *in vitro*

Elias A.-H. BAYDOUN and D. H. NORTHCOTE

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

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Membrane fusion *in vitro* between Golgi apparatus- and plasma-membrane-rich fractions isolated from maize (*Zea mays*) roots was found to be dependent on Ca^{2+} and the membrane proteins. Trypsin treatment of mixed membrane fractions before the addition of Ca^{2+} inhibited their ability to fuse. It resulted also in a selective and progressive elimination of a characteristic intense polypeptide band (B1) on gel electrophoresis. This polypeptide was not removed by chymotrypsin or thermolysin. B1 is an integral membrane protein with an exposed portion to the outside. Sodium deoxycholate was used to solubilize the proteins of mixed membrane fractions. Extracted proteins analysed by non-SDS (sodium dodecyl sulphate) polyacrylamide-gel electrophoresis revealed the presence of four isolated bands. When re-electrophoresed in the presence of SDS, one of these bands exhibited the same mobility as polypeptide B1. Enzymic staining of non-SDS-polyacrylamide gels showed that this protein has Ca^{2+} - and Mg^{2+} -dependent ATPase activity. Its possible role in membrane fusion is discussed.

The mechanism of membrane fusion is incompletely understood. Most of the information is based on electron-microscopic observations of intact cells inactive in secretion (Palade & Bruns, 1968; Satir *et al.*, 1973; Pinto da Silva & Nogueira, 1977) or on experiments with model liposome membranes (Papahadjopoulos, 1978).

Measurement of the transfer of radioactivity between radioactive Golgi-apparatus- and non-radioactive plasma-membrane-rich fractions isolated from maize (*Zea mays*) root tips has enabled us to develop a quantitative system to study membrane fusion *in vitro* (Baydoun & Northcote, 1980b). We have shown that the fusion process was dependent on the presence of Ca^{2+} . Our results suggested a possible role of protein in membrane fusion.

We report in the present paper the characterization of an integral membrane protein that might serve as a Ca^{2+} acceptor in membrane fusion. This protein has an ATPase activity that is stimulated in the presence of Ca^{2+} or Mg^{2+} . It is suggested that the ATPase has a structural role in membrane fusion *in vitro*.

Materials and methods

D-[U- ^{14}C]Glucose (sp. radioactivity 292 mCi/mmol), D-[1- ^3H]glucosamine (sp. radioactivity 2 Ci/mmol), L-[6- ^3H]fucose (sp. radioactivity 26 Ci/mmol) and [*methyl*- ^{14}C]choline chloride (sp. radioactivity 59 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Trypsin (EC 3.4.21.4, sp. activity 33 units/mg dry weight), chymotrypsin A₄ (EC 3.4.21.1, sp. activity 750 units/mg dry weight) and thermolysin (EC 3.4.24.4, sp. activity 40 units/mg dry weight) were obtained from Boehringer, Mannheim, Germany. Trypsin inhibitor from soya bean, and wheat-germ agglutinin, were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Trypsin was assayed as described by Baydoun & Northcote (1980b). Chymotrypsin was assayed spectrophotometrically at 237 nm by monitoring the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (Schwert & Takenaka, 1955) in the presence of 10 mM-Tris/HCl, pH 7.4, 0.1 mM-MgCl₂, 1 mM-EDTA and 34% (w/w) sucrose solution. Under these conditions, chymotrypsin (0.1 mg·ml⁻¹) caused a decrease in A_{237} of 0.34·min⁻¹ (at 0–4°C) and of 0.48·min⁻¹ (at 20°C). The activity of the enzyme was completely and instantaneously abolished by trypsin inhibitor at all the ratios tested

Abbreviations used: Ca^{2+} - and Mg^{2+} -ATPases, Ca^{2+} - and Mg^{2+} -dependent ATPases; SDS, sodium dodecyl sulphate.

(chymotrypsin/trypsin inhibitor 1:1, 1:1.5, 1:2, w/w, at 0–4°C or at 20°C).

Thermolysin was prepared and assayed by using a casein-digestion procedure (Matsubara, 1970) in the buffer described for the chymotrypsin assay. Incubation for 30 min at 0°C with thermolysin (0.1 mg·ml⁻¹) resulted in the liberation of material soluble in trichloroacetic acid that had an A_{280} of 0.37. The enzyme activity was lost completely with 10 mM-EDTA.

The ability of wheat-germ agglutinin to agglutinate erythrocytes was tested. A suspension of erythrocytes (50 μ l) prepared in phosphate-buffered saline (g/litre: Na₂HPO₄, 1.48; KH₂PO₄, 0.5; NaCl, 7.2; pH 7.4) was added to a serial dilution of wheat-germ agglutinin (50 μ l) made up in the buffer described for chymotrypsin assay. Under these conditions, wheat-germ agglutinin at all the concentrations used (1.25, 2.5, 5, 10 and 20 μ g·ml⁻¹) caused agglutination of the erythrocytes.

Growth of maize seedlings, incubation of roots with radioactive precursors, homogenization, membrane fractionation, and determination of radioactivity were performed as previously described (Baydoun & Northcote, 1980a).

Quantitative measurement of membrane fusion in vitro

Membrane fusion *in vitro* between radioactive Golgi-apparatus-rich and non-radioactive plasma-membrane-rich fractions was quantified by measuring the transfer of radioactivity between the mixed membrane fractions as described previously (Baydoun & Northcote, 1980b). Radioactive Golgi-apparatus-rich fraction (0.5 ml) was mixed with an equal volume of non-radioactive plasma-membrane-rich fraction at 30°C for 40 min in the presence of 1 mM-Ca²⁺ (final apparent free concentration) in a final volume of 1.04 ml. EDTA was then added to 6 mM. Mixed membranes were then re-fractionated by centrifugation (100 000 g, 4°C, 90 min) and the distribution of radioactivity in the fractions was determined.

Enzymic modification of the membranes was performed as follows. Mixed membrane fractions were pretreated at 0°C for 30 min with various concentrations of trypsin or chymotrypsin (1–100 μ g). Trypsin inhibitor [trypsin (or chymotrypsin)/trypsin inhibitor, 1:1.5, w/w] was then added. After thorough mixing, Ca²⁺ was added to 1 mM and the membranes were maintained at 30°C for 40 min and re-fractionated by centrifugation. Pretreatment with wheat-germ agglutinin was performed at 30°C for 30 min.

Extraction of membrane proteins

Extraction with KCl. Membrane fractions were mixed with KCl (final concn. 1, 2 or 3 M) and

sonicated (2 min at 10 s intervals). They were then left for 10 min at 4°C and centrifuged (100 000 g, 4°C, 1 h). The pellets were resuspended twice by sonication with 10 mM-Tris/HCl, pH 7.4, and re-centrifuged (100 000 g, 4°C, 30 min). The pellets were then processed for SDS/polyacrylamide-gel electrophoresis.

Extraction with pyridine. Membrane fraction (1 vol.) was mixed at 4°C with re-distilled pyridine (0.5 vol.) containing 0.3% mercaptoethanol (Blumenfeld & Zvilichovsky, 1972). The membrane fraction clarified instantaneously. The solution was then dialysed at 4°C for 16 h against several changes of water and centrifuged (90 000 g, 4°C, 90 min). The supernatant was freeze-dried and the pellet washed twice by sequential resuspension by sonication with 10 mM-Tris/HCl, pH 7.4, and centrifugation. The freeze-dried supernatant and the pellet were processed for SDS/polyacrylamide-gel electrophoresis.

Extraction with lithium di-iodosalicylate. Before its extraction with lithium di-iodosalicylate, a membrane fraction was suspended in the homogenization medium and centrifuged (100 000 g, 4°C, 30 min). The pellet was suspended in 0.5 ml of 0.3 M-lithium di-iodosalicylate prepared in 10 mM-Tris/HCl, pH 7.4, and stirred at room temperature for 5 min. The suspension was then mixed with 1 ml of water and stirred for a further 5 min at 4°C and centrifuged (45 000 g, 4°C, 90 min). The pellet was resuspended twice by sonication with 10 mM-Tris/HCl, pH 7.4, and re-centrifuged (100 000 g, 4°C, 30 min). The supernatant was dialysed overnight at 4°C against several changes of water and then freeze-dried. The freeze-dried supernatant and the pellet were processed for SDS/polyacrylamide-gel electrophoresis.

SDS/polyacrylamide gel electrophoresis

After they were pelleted (100 000 g, 4°C, 30 min), membrane fractions were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Samples were solubilized by heating for 5 min at 100°C in a sample buffer (200 μ l, 1–2 mg of protein·ml⁻¹) containing 0.77 g of dithiothreitol, 1.0 g of SDS, 4.0 ml of 1 M-Tris/HCl, pH 6.8, 5.0 ml of glycerol, 0.3 ml of Bromophenol Blue (0.2% in ethanol) and water up to 50 ml. The running gel contained 15% (w/v) polyacrylamide and was overlaid with 5%-acrylamide stacking gel. Gels were run in a Tris/glycine electrode buffer system at pH 8.3 containing 0.1% SDS at 100 V for 1 h and then at 200 V for 4 h. A mixture of marker proteins (each 5 μ g) was run in parallel on each gel. The mixture consisted of lysozyme (mol.wt. 14 300), creatine kinase (mol.wt. 40 000) and bovine serum albumin (mol.wt. 68 000).

After electrophoresis, gels to be stained for proteins were fixed in methanol/acetic acid/water

(9:2:9, by vol.) for 30 min, stained for a further 30 min with 0.2% Coomassie Brilliant Blue R made up in the fixer and destained in methanol/acetic acid/water (1:1:8, by vol.). Gels to be stained for glycoproteins were fixed as described above and stained with periodic acid/Schiff reagent (Fairbanks *et al.*, 1971) or by the thymol/H₂SO₄ method (Racusen, 1979). Radioactive slab gels were analysed by fluorography (Laskey & Mills, 1975).

Polyacrylamide-gel electrophoresis in the absence of SDS

Membranes were solubilized for 5 min at 20°C with stirring in a sample buffer (about 1–2 mg of protein·ml⁻¹) containing all the ingredients of that used for SDS/polyacrylamide-gel electrophoresis, except that SDS was omitted and replaced by sodium deoxycholate to a final concentration of 1%. The sample buffer was also modified by the addition of phenylmethanesulphonyl fluoride.

Samples were subjected to electrophoresis (running gel, 7.5% acrylamide and stacking gel, 3%), with a Tris/glycine electrode buffer system identical with that used for SDS/polyacrylamide-gel electrophoresis, but containing no SDS.

After electrophoresis (100 V for 1 h followed by 200 V for 3 h), gels were fixed, stained for proteins and destained.

Elution of proteins from SDS/polyacrylamide gels

Proteins were eluted from SDS/polyacrylamide gels by a modification of the method of Bray & Brownlee (1973) and analysed for their amino acid composition (Baydoun & Northcote, 1980a).

Staining polyacrylamide gels for enzymic activities

Polyacrylamide gels in the absence of SDS were assayed for ATPase activity (Wachstein & Meisel, 1957). The staining solution consisted of 0.1 M-Tris/maleate, pH 7.2, 8 mM-ATP, 3.5 mM-Pb(NO₃)₂ and 8 mM-MgCl₂ or CaCl₂. After incubation at 37°C for 1 h with gentle shaking, gels were washed thoroughly with water and immersed in a dilute (NH₄)₂S solution. Deposits of black PbS identified the sites of enzymic activity.

The staining solution for peroxidase consisted of a saturated solution of benzidine hydrochloride in 100 mM-sodium acetate buffer, pH 5.0, and 10 mM-H₂O₂. Phosphatase and esterase activities were made visible as described by Dulaney & Touster (1970).

Results

Proteolytic modification of the membrane fractions and its effect on membrane fusion in vitro

Radioactive Golgi-apparatus-rich fraction was mixed with non-radioactive plasma-membrane-rich

fraction in the presence of trypsin or chymotrypsin before the addition of Ca²⁺.

Trypsin treatment of the individual membrane fractions did not influence their behaviour on re-centrifugation (Baydoun & Northcote, 1980b). The transfer of radioactivity from radioactive Golgi-apparatus-rich fraction (obtained from maize roots incubated with D-[U-¹⁴C]glucose *in vivo*) to non-radioactive plasma-membrane-rich fraction was markedly inhibited by trypsin treatment (Fig. 1). Trypsin treatment resulted also in the loss of radioactivity. After re-fractionation, the total amount of radioactivity recovered was 94.7% (heat-inactivated enzyme), 91.0% (1 µg of trypsin·ml⁻¹), 78.9% (10 µg of trypsin·ml⁻¹), 79.1% (31.6 µg of trypsin·ml⁻¹) and 69.6% (100 µg of trypsin·ml⁻¹). This indicated that radioactive proteins were removed as a result of trypsin treatment. In another experiment, radioactive Golgi-apparatus-rich fraction (obtained from roots incubated with

Fig. 1. Effect of trypsin on the transfer of radioactivity between mixed membrane fractions

Roots were incubated with D-[U-¹⁴C]glucose *in vivo*. Golgi-apparatus-rich fraction was prepared from radioactive roots. Plasma membrane-rich fraction was prepared from non-radioactive roots. These were mixed at 0°C for 30 min in the presence of various concentrations of trypsin (●); trypsin inhibitor (trypsin/trypsin inhibitor, 1:1.5, w/w) was then added. After thorough mixing, Ca²⁺ was added to 1 mM and the membranes were maintained at 30°C for 40 min and re-fractionated by centrifugation. The radioactivity transferred to the plasma membrane-rich fraction was determined. In the control (○), heat-inactivated enzyme (100 µg·ml⁻¹) was used.

[methyl- ^{14}C]choline chloride *in vivo*) was mixed with non-radioactive plasma-membrane-rich fraction. Trypsin treatment inhibited the transfer of radioactivity (Table 1) in a manner similar to that obtained when D-[U- ^{14}C]glucose was used as a radioactive precursor; however, the total amount of radioactivity recovered was not affected by the trypsin treatment.

Table 1. *Effect of trypsin on the transfer of radioactive phosphatidylcholine between mixed membrane fractions*

Roots were incubated with [methyl- ^{14}C]choline chloride *in vivo*. Golgi-apparatus-rich fraction was prepared from radioactive roots. Plasma membrane-rich fraction was prepared from non-radioactive roots. These were mixed at 0°C for 30 min in the presence of trypsin (31.6 $\mu\text{g}\cdot\text{ml}^{-1}$); trypsin inhibitor (trypsin/trypsin inhibitor, 1:1.5, w/w) was then added. After thorough mixing, Ca^{2+} was added to 1 mM and the membranes were maintained at 30°C for 40 min and re-fractionated by centrifugation. The distribution of radioactivity in the fractions was determined. In the control, heat-inactivated enzyme was used.

Fraction	Radioactivity (%) from re-fractionated membranes after mixing	
	Trypsin-treated membranes	Control
Golgi apparatus-rich	81.2	52.2
Plasma membrane-rich	16.0	45.9
Remainder fraction	2.8	1.9
Total radioactivity (c.p.m.)	8860	8700
obtained from all the fractions		
Recovery	95.1	93.4

Similar effects of trypsin on the transfer of radioactivity were obtained when individual radioactive membrane fractions were treated with trypsin before mixing with untreated non-radioactive membrane fractions (Table 2).

The gel patterns of the various isolated membrane fractions revealed two characteristic intense bands (B1 and B2, Fig. 2), of mol.wt. of about 36000 and 35000, and several other bands of different mobilities. Although B1 and B2 were present in all the isolated membrane fractions, they were more intense in the Golgi-apparatus- and plasma-membrane-rich fractions.

A total homogenate of maize root tips was centrifuged at 100000 g for 1 h and at 4°C. The supernatant was dialysed against several changes of water at 4°C for 6 h and freeze-dried; the pellet was washed twice by sequential centrifugation and resuspension by sonication in 10 mM-Tris/HCl, pH 7.4. On SDS/polyacrylamide-gel electrophoresis, bands B1 and B2 appeared in the pellet as well as in the total homogenate fraction (Fig. 3), but they were absent from the freeze-dried supernatant; therefore the polypeptides B1 and B2 were probably membrane-bound proteins.

Mixed Golgi-apparatus- and plasma-membrane-rich fractions were treated with increasing concentrations of trypsin and subjected to SDS/polyacrylamide-gel electrophoresis. A selective and progressive elimination of the polypeptide B1 occurred (Fig. 4). Other polypeptides were not affected by trypsin treatment under the incubation conditions. Two bands of lower molecular weight appeared in the trypsin-treated fractions; these were absent from the control. They could represent the products of trypsin digestion, as the washing procedure removed all the trypsin and trypsin inhibitor added.

Table 2. *Trypsin treatment of individual membrane fractions and its effect on the transfer of radioactivity*

Roots were incubated with D-[U- ^{14}C]glucose *in vivo*. Membrane fractions were prepared from radioactive (*) and non-radioactive (†) roots. Radioactive Golgi-apparatus- and plasma-membrane-rich fractions were treated individually with trypsin (31.6 $\mu\text{g}\cdot\text{ml}^{-1}$) at 0°C. After 30 min, trypsin inhibitor was added (trypsin/trypsin inhibitor, 1:1.5, w/w). Trypsin-treated Golgi-apparatus-rich fraction (GA*) was mixed with non-radioactive plasma-membrane-rich fraction (PM†); alternatively, trypsin-treated plasma-membrane-rich fraction (PM*) was mixed with non-radioactive Golgi-apparatus-rich fraction (GA†). The mixtures were maintained at 30°C for 40 min in the presence of 1 mM- Ca^{2+} and then re-fractionated by centrifugation. The distribution of radioactivity in the fractions was determined. In the control, heat-inactivated enzyme was used.

Fraction	Radioactivity (%) from re-fractionated membranes after mixing:			
	Trypsin-treated GA* with PM†	Control	Trypsin-treated PM* with GA†	Control
Golgi-apparatus-rich	76.9	48.8	24.7	42.4
Plasma-membrane-rich	21.3	49.9	74.0	56.1
Remainder fraction	1.8	1.3	1.3	1.5
Total radioactivity (c.p.m.) obtained from all the fractions	16450	19320	9590	10660
Recovery	75.7	88.9	82.7	91.9

Fig. 2. Electrophoresis on SDS/15%-polyacrylamide gel of the isolated membrane fractions

Track 1, endoplasmic-reticulum-rich fraction; track 2, Golgi-apparatus-rich fraction; track 3, plasma-membrane-rich fraction; track 4, mitochondria-rich fraction (samples containing approx. 100 μ g of protein were applied per track); track 5, protein markers with molecular weights as indicated. Staining was with Coomassie Brilliant Blue.

Mixed membrane fractions were incubated with chymotrypsin (0.1 mg $\cdot$ ml⁻¹) or thermolysin (0.1 mg $\cdot$ ml⁻¹) at 0°C for 30 min and electrophoresed on SDS/polyacrylamide gel. The pattern of separation of polypeptides was not affected by thermolysin treatment, it was identical with that of the control. Chymotrypsin treatment resulted in the loss of some bands of high molecular weight. Both enzymes had no effect on any of the polypeptides B1 or B2.

Chymotrypsin treatment, in common with trypsin treatment of the individual membrane fractions (Baydoun & Northcote, 1980b), did not influence their behaviour on re-centrifugation. Radioactive Golgi-apparatus-rich fraction was mixed with non-radioactive plasma-membrane-rich fraction and pre-treated with chymotrypsin (0.1 mg $\cdot$ ml⁻¹) at 0°C for 30 min and then with trypsin inhibitor before the addition of Ca²⁺ and further incubation at 30°C for 40 min. A decrease in the transfer of radioactivity was observed as a result of chymotrypsin treatment (Table 3). Chymotrypsin was not as effective as

Fig. 3. SDS/polyacrylamide-gel electrophoresis of a total homogenate of maize root tips (track 1), 100 000 g supernatant (track 2) and 100 000 g pellet (track 3). Approx. 150 μ g of protein was applied per track. Protein markers with molecular weights as indicated are shown in track 4. The total homogenate of maize root tips was centrifuged (100 000 g, 4°C, 1 h). The washed pellet, the dialysed supernatant and the dialysed total homogenate were analysed on an SDS/15%-polyacrylamide gel. Staining was with Coomassie Brilliant Blue.

trypsin in inhibiting the transfer of radioactivity between mixed membrane fractions. The amount of radioactivity recovered from all the fractions after incubation in the presence of Ca²⁺ (63.1%) was less than that found after the addition of trypsin inhibitor (75.2%). However, the addition of Ca²⁺ re-activated the enzyme, even in the presence of the inhibitor (approx. 10% of the original activity at 20°C), so that further loss of radioactivity was to be expected. Even after the continued digestion at 30°C, the polypeptide B1 was not removed, although a large number of other polypeptides were lost (Fig. 5).

Characterization of the polypeptide bands B1 and B2

Glycoprotein identification. SDS/polyacrylamide gels of mixed Golgi-apparatus- and plasma-mem-

Fig. 4. SDS/polyacrylamide-gel electrophoresis of the trypsin-treated mixed membrane fractions

Track 1, mixed membranes with no trypsin added; tracks 2–5, mixed membranes in the presence of the following concentrations of trypsin: $1\mu\text{g}\cdot\text{ml}^{-1}$ (2), $10\mu\text{g}\cdot\text{ml}^{-1}$ (3), $31.6\mu\text{g}\cdot\text{ml}^{-1}$ (4) and $100\mu\text{g}\cdot\text{ml}^{-1}$ (5) (approx. $100\mu\text{g}$ of protein was applied per track); track 6, trypsin and trypsin inhibitor ($10\mu\text{g}$ each); track 7, protein markers with molecular weights as indicated. Golgi-apparatus- and plasma-membrane-rich fractions were prepared from maize roots and mixed at 0°C for 30 min in the presence of various concentrations of trypsin. Trypsin inhibitor was then added (trypsin/trypsin inhibitor, 1:1.5, w/w) and the membranes were washed twice by sequential centrifugation and resuspension by sonication in 10 mM-Tris/HCl, pH 7.4. The final pellets were analysed on an SDS/15%-polyacrylamide gel. Staining was with Coomassie Brilliant Blue.

brane-rich fractions were stained for glycoproteins, and gels run in parallel were also prepared for the detection of proteins. No bands stained positively for glycoproteins when both stains were tested. A positive stain appeared only at the top and at the front of the gel. This may be due to phosphatidylinositol and glycolipid constituents of the membrane. However, the sensitivity of the stains was not adequate enough to detect small amounts of glycoproteins; glycoprotein markers (e.g. ovomucoid) stained positively only if they were applied in large quantities ($10\text{--}20\mu\text{g}$).

Maize roots (100) were incubated *in vivo* with L-[6- ^3H]fucose ($100\mu\text{Ci}$) or D-[1- ^3H]glucosamine

Table 3. Effect of chymotrypsin on the transfer of radioactivity between mixed membrane fractions

Roots were incubated with D-[U- ^{14}C]glucose *in vivo*. Golgi-apparatus-rich fraction was prepared from radioactive roots. Plasma-membrane-rich fraction was prepared from non-radioactive roots. These were mixed at 0°C for 30 min in the presence of chymotrypsin ($100\mu\text{g}\cdot\text{ml}^{-1}$); trypsin inhibitor (chymotrypsin/trypsin inhibitor, 1:1.5, w/w) was then added. After thorough mixing, Ca^{2+} was added to 1 mM and the membranes were maintained at 30°C for 40 min and re-fractionated by centrifugation. The distribution of radioactivity between the fractions was determined. In the control, heat-inactivated enzyme was used.

Fraction	Radioactivity (%) from fractionated membranes after mixing:	
	Chymotrypsin-treated membranes	Control
Golgi-apparatus-rich	66.2	48.1
Plasma-membrane-rich	33.0	50.6
Remainder fraction	0.8	1.3
Total radioactivity (c.p.m.) obtained from all the fractions	10850	15320
Recovery	63.1	89.0

(1 mCi) for 1 h by the same method as that with radioactive glucose (Baydoun & Northcote, 1980a). After membrane fractionation, mixed Golgi-apparatus- and plasma-membrane-rich fractions were electrophoresed on SDS/polyacrylamide gels. Gels were stained with Coomassie Blue for proteins and destained (Fig. 6b). They were then fluorographed for analysis of radioactive proteins (Fig. 6a). Comparison of the relative mobilities of polypeptides B1 and B2 in the stained gel with those in the fluorographed gel showed that radioactivity from both precursors was not incorporated into polypeptide B1; however, there was a faint indication that radioactivity from glucosamine could be present in B2.

Radioactive Golgi-apparatus-rich fraction was mixed with non-radioactive plasma-membrane-rich fraction in the presence of various concentrations of wheat-germ agglutinin (1.25 , 2.5 , 5 , 10 and $20\mu\text{g}\cdot\text{ml}^{-1}$) at 30°C for 30 min before the addition of Ca^{2+} (1 mM) and further incubation for 40 min. Although the concentrations of wheat-germ agglutinin used in the present investigation were able to agglutinate erythrocytes, the transfer of radioactivity between mixed membrane fractions was not affected.

Extraction of membrane proteins. SDS/polyacrylamide-gel electrophoresis of mixed membrane

Fig. 5. SDS/polyacrylamide-gel electrophoresis of mixed membranes treated with chymotrypsin under the conditions devised for membrane fusion

Track 1, mixed membranes in the presence of heat-inactivated enzyme; track 2, mixed membranes in the presence of chymotrypsin (approx. 100 μ g of protein was applied per track); track 3, protein markers with molecular weights as indicated. Golgi-apparatus- and plasma-membrane-rich fractions were prepared from maize roots and mixed at 0°C for 30 min in the presence of chymotrypsin (0.1 mg·ml⁻¹). Trypsin inhibitor was then added (chymotrypsin/trypsin inhibitor, 1:1.5, w/w). After thorough mixing, Ca²⁺ was added to 1 mM and the membranes were maintained at 30°C for 40 min. Membranes were then washed twice by sequential centrifugation and resuspension by sonication in 10 mM-Tris/HCl, pH 7.4. The final pellets were analysed on an SDS/15%-polyacrylamide gel. In the control, heat-inactivated enzyme was used. Staining was with Coomassie Brilliant Blue.

peptide B1 nor B2 was extracted by KCl treatment. Pyridine extracted most of the mixed membrane proteins. This extraction was not complete, since most of the membrane proteins were demonstrated in the membrane pellet left after extraction. The most effective reagent was lithium di-iodosalicylate (0.3 M); it resulted in almost complete extraction of membrane proteins.

Elution of polypeptides B1 and B2 from gels and their amino acid composition. After elution, each protein (B1 and B2) appeared as a single band on SDS/polyacrylamide-gel electrophoresis. The amino acid composition of both polypeptides did not differ considerably from that of mixed Golgi-apparatus- and plasma-membrane-rich fractions (Table 4).

Non-SDS polyacrylamide-gel-electrophoretic patterns of the proteins of mixed membrane fractions. Mixed Golgi-apparatus- and plasma-membrane-rich fractions were centrifuged (100 000 g, 4°C, 30 min) and the pellet washed twice by sequential centrifugation and resuspension in 10 mM-Tris/HCl, pH 7.4. The pellet was extracted in a sample buffer containing 1% sodium deoxycholate and electrophoresed on non-SDS 7.5% polyacrylamide gel. Staining of the gel with Coomassie Blue revealed the presence of four bands (a, b, c and d; Fig. 7). Although the resolution of bands a and b on the gel was poor, band a was more intensely stained than b. Most of the membrane proteins did not penetrate the gel; they were present along the first part of the stacking gel or running gel (Fig. 7a).

The incorporation of sodium deoxycholate to a final concentration of 0.1% in the electrode buffer and in the running and stacking gels gave a poorer resolution of bands a and b (Fig. 7b). This disagrees with the results of Dulaney & Touster (1970). Therefore deoxycholate was omitted from the gels and the electrode buffer. Deoxycholate precipitates in the gel in the presence of 10% acetic acid and other fixatives, forming a diffuse background against which it is difficult to discern faint bands of protein after Coomassie Blue staining.

Re-electrophoresis of non-SDS gel on SDS/15%-polyacrylamide gel

Membrane proteins dissolved in deoxycholate were separated electrophoretically on non-SDS 7.5%-polyacrylamide gel. After electrophoresis they were stained with 0.2% Coomassie Blue for 10 min; as soon as the bands were just stained, the gel was washed several times with water. The areas containing bands a, b, c and d as well as the first parts of the stacking and running gels were cut from two tracks and soaked for 30 min in the SDS-containing sample buffer. Gel pieces were then transferred to the wells of a previously prepared SDS/15%-polyacrylamide gel and run electrophoretically. At the end of electrophoresis, gels were stained with Coomassie

fractions treated with KCl showed that some proteins were extracted. Increasing the KCl concentration above 2 M did not result in further extraction of membrane proteins. Neither poly-

Blue. The results are shown in Fig. 8. Bands a and b of the non-SDS polyacrylamide gel had the same mobilities as polypeptides B1 and B2 identified on SDS/polyacrylamide gels. However, both bands were cross-contaminated. This could be due to the poor separation of these bands on the non-SDS polyacrylamide gel; alternatively, it could reflect a structural or functional relationship between these bands. The results also show that deoxycholate resulted in almost complete extraction of the polypeptide B1 compared with the partial extraction of the polypeptide B2. Most of the polypeptide B2 was left at the first part of the non-SDS polyacrylamide stacking gel (Fig. 8).

Table 4. *Amino acid composition of polypeptides B1 and B2 and of the mixed membrane fractions*

Samples were hydrolysed with 6M-HCl in an evacuated sealed tube at 105°C for 24 h. The hydrolysed samples were dried, dissolved in citrate buffer and assayed for amino acid composition.

Amino acid	Composition (residues/100 residues)		
	Polypeptide B1	Polypeptide B2	Mixed membranes
Asp	9.9	9.5	9.0
Thr	4.9	4.6	5.1
Ser	6.8	5.9	6.9
Glu	9.9	9.7	9.5
Pro	5.5	5.1	5.5
Gly	10.3	10.8	9.8
Ala	9.2	9.7	11.1
Val	5.9	7.6	7.6
Met	0.9	1.2	1.0
Ile	3.6	3.6	3.9
Leu	8.3	7.7	8.3
Tyr	3.2	3.0	2.9
Phe	4.8	5.4	5.5
His	2.9	3.8	2.5
Lys	6.2	5.8	5.6
Arg	7.8	7.0	5.9

Fig. 6. *Electrophoresis on SDS/15%-polyacrylamide gel of radioactive mixed membrane fractions as analysed by fluorography (a) or Coomassie Blue staining (b)*

Track (a)1, mixed membranes prepared from roots incubated with D-[1-³H]glucosamine (exposed for 12 h); track (a)2, mixed membranes prepared from roots incubated with L-[6-³H]fucose (exposed for 48 h); track (a)3, radioactive protein markers containing: i, myosin (mol.wt. 200 000); ii, phosphorylase b (92 500); iii, bovine serum albumin (68 000); iv, ovalbumin (46 000); v, carbonic anhydrase (30 000); and vi, lysozyme (14 300). Tracks (b)1 and 2 are the Coomassie Blue-stained images of tracks (a)1 and (a)2 respectively. Approx. 100 µg of protein was applied per track. Track (b)3, protein markers with molecular weights as indicated.

Fig. 7. Non-SDS polyacrylamide-gel electrophoresis of the mixed membranes

(a) No deoxycholate was incorporated into the gel or electrode buffer; (b) deoxycholate was incorporated into the gel and electrode buffer. Golgi-apparatus- and plasma-membrane-rich fractions were prepared from maize roots. They were mixed and pelleted. The pellets were washed by sequential centrifugation and resuspension by sonication in 10mM-Tris/HCl, pH 7.4. The final pellets were solubilized in a sample buffer containing 1% sodium deoxycholate and analysed on a non-SDS 7.5%-polyacrylamide gel. Staining was with Coomassie Brilliant Blue.

Enzymic staining of polyacrylamide gels

Polyacrylamide gels in the absence of SDS were stained for the activities of ATPase, phosphatase, esterase and peroxidase.

Fig. 8. Electrophoresis on SDS/15%-polyacrylamide gel of the proteins from a non-SDS polyacrylamide gel. Track 1, mixed membranes (about 100µg of protein); tracks 2, 3, 4 and 5, bands a, b, c and d respectively of the non-SDS gel (Fig. 7); tracks 6 and 7, the first parts of the stacking and running gels of the non-SDS gel respectively; track 8, protein markers with molecular weights as indicated. Staining was with Coomassie Brilliant Blue.

Fig. 9. Mixed membranes solubilized with sodium deoxycholate, analysed on a non-SDS/7.5%-polyacrylamide gel and stained for ATPase (a) and peroxidase (b) activities

Track (a)1, ATPase activity in the absence of Ca²⁺ or Mg²⁺; track (a)2, ATPase activity in the presence of Ca²⁺; track (a)3, ATPase activity in the presence of Mg²⁺; track (b)1, peroxidase activity; track (b)2, a crude preparation of horseradish peroxidase (10µg) run and stained under the same conditions.

Staining procedure for all these enzymes showed that some activity was left at the top of stacking or running gels. This could be due to material that was unable to penetrate the gel during electrophoresis, possibly owing to the formation of large aggregates that are tightly bound to some particulate material in the deoxycholate extract.

Esterase and phosphatase were not detected in the running gel. Esterase was present as a very faint band at the top of the stacking gel. Phosphatase activity was localized at the top of the stacking gel and to a lesser extent at the top of the running gel. Acid phosphatase (EC 3.1.3.2; Boehringer) run under the same conditions was detected.

Bands a and b identified by Coomassie Blue showed enzymic activity when stained for ATPase. Lead sulphide deposits were located almost exclusively in these bands (Fig. 9a). A requirement for Ca^{2+} and for Mg^{2+} for the enzyme activity was shown; in the absence of these bivalent cations, very little activity was detected.

None of the separated bands (a, b, c and d) showed peroxidase activity (Fig. 9b). A crude preparation of horseradish peroxidase (EC 1.11.1.7, Sigma) run under the same conditions was detected (Fig. 9b).

Discussion

The present work has shown that modification of the mixed membrane fractions by treatment with trypsin before the addition of Ca^{2+} inhibited the ability of the membranes to fuse. Similar effects were obtained when one fraction of membrane vesicles was treated with trypsin and mixed with an untreated sample of membrane vesicles.

Experiments with radioactive glucose showed that the inhibition in the transfer of radioactivity, which was a measure of membrane fusion, due to trypsin treatment was accompanied by a decrease in the recovery of the total amount of radioactivity. Radioactive glucose was found to label all the membrane constituents, including proteins, lipids and carbohydrates (Baydoun & Northcote, 1980a). The use of [*methyl*- ^{14}C]choline chloride as a radioactive label excluded the possibility that the inhibition in the transfer of radioactivity was due to the loss of radioactive proteins after trypsin treatment; radioactive choline chloride was found to label phosphatidylcholine constituent of the membranes only (Baydoun & Northcote, 1980a).

The results indicated that Ca^{2+} could probably form bridges between mixed membrane vesicles through negative charges on surface proteins or glycoproteins. These bridges would bring membranes into close contact, a prerequisite for membrane fusion. The selective loss of one of the polypeptides (B1) of the mixed membrane fractions

after trypsin treatment indicated a possible relationship between this polypeptide and the sites of Ca^{2+} binding.

Trypsin treatment did not result in complete elimination of the polypeptide. The reason for this is possibly that during homogenization two types of vesicles are produced (right-side-out and inside-out); the enzyme has access to the vesicles with the protein exposed externally. It is also possible that the band on the gel represents more than one polypeptide. The polypeptide was not removed by chymotrypsin or thermolysin treatment, which indicated the lack of susceptible bonds to these enzymes in the externally accessible portion of the membrane protein.

Trypsin treatment did not completely abolish membrane fusion in the presence of Ca^{2+} as measured by transfer of radioactivity. This could be due to the presence of other proteins that were not removed by trypsin. The results obtained with chymotrypsin also suggest that proteins other than the polypeptide B1 could bind Ca^{2+} . However, it was possible that Ca^{2+} could bind to acidic phospholipids to bring membranes in contact. This seems to be true for artificial lipid membranes (Papahadjopoulos, 1978). Our findings are consistent with results obtained from other studies of the effect of trypsin treatment on Ca^{2+} -induced membrane fusion (Gratzl & Dahl, 1978; Dahl *et al.*, 1978; Sandra, 1980).

Membranes of isolated secretory vesicles and myoblasts (Gratzl & Dahl, 1978; Dahl *et al.*, 1978) treated with neuraminidase have been shown to exhibit decreased ability for fusion, so that there might possibly be a role for glycoproteins during the process. However, it is difficult to draw any general conclusions from these results, since fusion of erythrocytes was found to be facilitated by pretreatment with neuraminidase (Ahkong *et al.*, 1973), and there was no effect of neuraminidase on the entry of Ca^{2+} into erythrocytes in the presence of fusogenic reagents, which again suggest that binding of Ca^{2+} to externally located sialic acid residues was unlikely (Blow *et al.*, 1979). In the present work, treatment of mixed membranes with wheat-germ agglutinin did not affect their ability to fuse. Wheat-germ agglutinin is a plant lectin and it is known to bind the free residues of *N*-acetyl-D-glucosamine and sialic acid of a variety of glycoproteins (Bhavanandan & Katlic, 1979).

Radioactive D-glucosamine and L-fucose were incorporated into some of the protein constituents of mixed membranes, but not into polypeptide B1, which was therefore unlikely to be a glycoprotein containing *N*-acetylglucosamine or fucose residues. It has been suggested that D-glucosamine is a specific precursor of amino-sugar components of glycoproteins (Roberts & Pollard, 1975). Fucose,

which is a marker for secreted slime in maize roots because it is not metabolized to other sugars (Kirby & Roberts, 1971), was also shown to be incorporated into some membrane glycoproteins (Green & Northcote, 1979).

The polypeptides B1 and B2 are not peripheral proteins. They were not released from the membranes by increasing the ionic strength with KCl (1–3 M). It is unlikely that these proteins would adhere to the membranes during homogenization; EDTA used in the homogenization and fractionation media would prevent such adhesion. In addition, the proteins present in the supernatant (100 000 g) did not contain either polypeptide B1 or B2. Pyridine and lithium di-iodosalicylate disrupt the lipid bilayer and dissolve integral membrane proteins. Both reagents extracted polypeptides B1 and B2. Kawai & Spiro (1980) and Nakamura *et al.* (1980) obtained almost complete solubilization of membrane proteins with 0.3 M-lithium di-iodosalicylate, by using the procedure described by Marchesi (1972). These results taken together with those obtained from the treatment with trypsin suggest that the polypeptide B2 is an integral protein buried in the membrane, whereas the polypeptide B1 is an integral protein with an exposed portion, possibly hydrophilic, to the outside.

Attempts were made to isolate and characterize the polypeptides B1 and B2 in native form. They were dissolved with detergents for subsequent analysis on polyacrylamide gels in the absence of SDS. During the present study, Triton X-100 and Nonidet P40 (non-ionic detergents) dissolved the membrane proteins, but no successful separation was achieved subsequently on polyacrylamide gels, even when these detergents were incorporated into the gels. Sodium deoxycholate (1%), an anionic detergent, solubilized the membrane proteins and allowed identification of enzymic activities on the polyacrylamide gels. Lower concentrations of deoxycholate (0.1–0.5%) were ineffective. Higher concentrations of deoxycholate could denature the enzymes and produce an irreversible loss of function (Korenbrodt, 1977).

In the present investigation, the only enzymic activity extracted and identified within the gel was that of ATPase, which showed Ca²⁺- and Mg²⁺-dependence. Deoxycholate has been used to extract ATPase from membranes of many different cells (Dulaney & Touster, 1970; Munoz *et al.*, 1970; Philippot, 1971; Malpartida & Serrano, 1980; Melgunov & Akimova, 1980). Most ATPases, including those isolated and purified, show Ca²⁺- and Mg²⁺-dependence even when they are dissociated into monomeric forms (Kawaguchi & Konishi, 1980; Møller *et al.*, 1980). Wheeler & Humphreys (1979) showed that Ca²⁺ was as effective as Mg²⁺ in activating the maize scutellum

ATPase in the intact tissue. However, Benson & Lipton (1978) purified a K⁺-stimulated ATPase from microsomal fractions isolated from maize roots by extraction of the membranes with NaClO₄. The purified enzyme had a mol.wt. of 30 500. Ca²⁺ had no effect on the purified enzyme, although it inhibited the enzyme activity in the microsomal fractions. It is possible that ATPase could undergo modification during homogenization and that such modification could affect its ionic requirement.

In our work, it is not clear whether the ATPase activity is due to both proteins a and b, because of the poor resolution of the two bands on the non-SDS-containing gel. However, these proteins showed similar characteristics to the polypeptides B1 and B2 when re-electrophoresed in the presence of SDS, and they might represent an enzyme complex. Their close molecular weights also suggests that B1 may be converted to B2 by the loss of a small peptide of mol.wt. of about 1000, possibly during homogenization and fractionation. Haddock & Jones (1977) stated that all the purified bacterial ATPases that have so far been examined can be dissociated into at least two non-identical subunits when subjected to SDS/polyacrylamide-gel electrophoresis. Some ATPases consist of two functional parts, one associated with ATP hydrolysis, the other involved in cation transport (Wheeler *et al.*, 1979).

Almost all cell membranes contain ATPases. They are usually Ca²⁺-binding proteins; the activity of the enzyme can be correlated with the transport of Ca²⁺, thus regulating the intracellular Ca²⁺ concentration. However, not all membrane ATPases are necessarily involved in Ca²⁺ transport (Kretsinger, 1976). (Ca²⁺ + Mg²⁺)-ATPase isolated from animal cells is susceptible to trypsin treatment. In pig and bovine erythrocyte membranes the loss of ATPase activity as a result of trypsin digestion was accompanied by a decrease in the Ca²⁺-binding activity of the isolated membranes (Kawaguchi & Konishi, 1980).

In the present study, the molecular weight of the maize root membrane ATPase was estimated to be about 36 000 by SDS/polyacrylamide-gel electrophoresis, similar to that found by Benson & Lipton (1978). The membrane ATPase of *Streptococcus faecalis* has a similar molecular weight and it is made of two major subunits each with a mol.wt. of 33 000 (Schnebli *et al.*, 1970). The ATPase of bovine heart mitochondria has a subunit mol.wt. of 29 000 (Peneffsky & Warner, 1965).

The secretion of histamine in mast cells is known to be dependent on the presence of external Ca²⁺. The removal of Ca²⁺-ATPase from the plasma membrane of these cells inhibited their ability to secrete (Cooper & Stanworth, 1977). The addition of dimethyl sulphoxide to sarcoplasmic-reticulum vesicles, isolated from rabbit skeletal muscle, pro-

moted their fusion. It was suggested that the dimethyl sulphoxide acted on the ATPase, which makes up about 80% of the protein of the reticulum (Stromer *et al.*, 1976). Poste & Allison (1973) in a theoretical scheme for membrane fusion, indicated that a membrane-bound Ca^{2+} -ATPase could possibly be involved.

The membrane ATPase that we have characterized from maize root cells has similar properties to bacterial and animal ATPases. It is possible that the role of this enzyme in the membrane fusion of Golgi-apparatus- and plasma-membrane-rich fractions *in vitro* is non-enzymic. No ATP was used in the fusion experiments. The loss of the enzyme by trypsin treatment correlated with the inhibition of membrane fusion; this indicated that Ca^{2+} binding to ATPase at the surface of the membrane vesicle is the first event that leads to membrane fusion *in vitro*. Ca^{2+} could affect the fusion by inducing further conformational changes in the membrane or by simply providing a bridge between the membranes.

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