Activity of phosphatidylcholine-transfer protein from spinach (*Spinacia oleracea*) leaves with mitochondria and chloroplasts

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A low-molecular-weight protein catalysing the transfer of phosphatidylcholine from liposomes to mitochondria and chloroplasts has been isolated from spinach (*Spinacia oleracea*) by chromatography on Sephadex G-75.

The existence of phosphatidylcholine-transfer proteins has been demonstrated in photosynthetic tissues such as spinach (Julienne *et al.*, 1980; Yamada *et al.*, 1980; Julienne & Kader, 1981) or *Avena* (Tanaka *et al.*, 1980) leaves. However, these experiments were performed on crude protein extracts containing not only phospholipid-transfer proteins but also lipases that could interfere with the transfer assay (Kader, 1975). This may explain why it was recently concluded that phospholipid-transfer proteins were lacking in total protein extracts prepared from spinach leaves (Murphy & Kuhn, 1981). It was necessary to eliminate these interfering components to try to explain this discrepancy.

The present paper shows that a single gel-filtration step is sufficient to get from spinach cytosol a stable phosphatidylcholine-transfer fraction, active with mitochondria and chloroplasts.

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Experimental

Materials

Sephadex G-75 was obtained from Pharmacia, Bois d'Arcy, France. [$Me-^3H$]Choline chloride and cholesteryl [$1-^{14}C$]oleate were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were Analytical-Reagent grade.

Methods

Cytosolic protein extracts were prepared from leaves excised from spinach (*Spinacia oleracea* var. Monstrueux de Viroflay) plants grown in a greenhouse or in nearby plots. Leaves (2.5 kg) were ground in 2 litres of 100 mM-Tris/HCl/5 mM-EDTA/400 mM-sucrose/1 mM-mercaptoethanol/5 mM-MgCl₂/1 mM-phenylmethanesulphonyl fluoride/

3 mM-sodium azide (pH 7.5). After centrifugation of the homogenate at 10000 g for 15 min, the mixture was centrifuged at 10000 g for 15 min and the green pellets, containing the residual membranes, were discarded. After adjustment of the pH to 7.5, 52 g of (NH₄)₂SO₄ was added per 100 ml of supernatant. After 3 h of stirring, the brown protein pellets were collected by centrifugation at 10000 g for 30 min and suspended in 50 mM-Tris/HCl/1 mM-EDTA/1 mM-phenylmethanesulphonyl fluoride/3 mM-sodium azide (pH 7.5) (Tris/EDTA buffer). The suspension was dialysed overnight against 20 vol. of Tris/EDTA buffer. After centrifugation at 10000 g for 20 min, about 250 ml of brown clear suspension containing 15 mg of protein per ml [determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard] was obtained. The suspension was concentrated with poly(ethylene glycol) (M_r 20000; Sigma, U.S.A.) (25 mg of protein/ml) and the concentrated non-diffusible solution (2 g of protein) was loaded on to a Sephadex G-75 column (80 cm × 5 cm) equilibrated with Tris/EDTA buffer. Proteins were eluted from the column with Tris/EDTA buffer at a flow rate of 80 ml/h and 16 ml fractions were collected.

Intact mitochondria were isolated from potato tubers by sucrose gradient by the method of Yamada *et al.* (1978) and suspended in Tris/EDTA buffer containing 250 mM-sucrose to give a concentration of 10 mg of protein/ml. Chloroplasts were isolated from spinach leaves by the method of Mills & Joy (1980) by the use of Percoll and suspended in Tris/EDTA buffer, containing 330 mM-sorbitol and 1 mM-MgCl₂, to give a concentration of 12 mg of protein/ml. Of the chloroplasts prepared by this method 90–95% were intact as determined by the ferricyanide test described by Lilley *et al.* (1975) and contained negligible succinate:cytochrome c

oxidoreductase (succinate dehydrogenase) activity (a mitochondrial enzyme) measured by the method of Douce *et al.* (1972).

Liposomes, prepared from [^3H]phosphatidylcholine $[(1.5\text{--}2.8) \times 10^4 \text{ d.p.m.}; 350 \text{ nmol/ml}]$ and cholesteryl [^{14}C]oleate ($2.0 \times 10^4 \text{ d.p.m./ml}$), were sonicated for 30 min at $15\text{--}20^\circ\text{C}$ in Tris/EDTA buffer (Julienne *et al.*, 1980). [^3H]Phosphatidylcholine was extracted from potato tuber slices incubated with [^3H]choline (Julienne *et al.*, 1980).

For transfer assays, 0.2 ml of a mitochondrial or chloroplast suspension (2 mg of protein) was incubated in polyethylene scintillation vials with 0.5 ml of liposomes and 3 ml of Tris/EDTA buffer or fractions from the G-75 column in a total volume of 3.7 ml for 60 min at 30°C . Sorbitol was added to the mixture to give a concentration of 330 mM. At the end of the incubation, the vials were centrifuged at 15 000 g for 10 min (for mitochondria) or 5000 g for 5 min (for chloroplasts) (SA-600 rotor; Sorvall, U.S.A.). The mitochondrial pellets were suspended in 0.3 ml of 1% Triton X-100. The radioactivity was determined in the same vial by adding 10 ml of toluene and Triton X-100 (2:1, v/v) containing 0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene as a scintillation mixture. The lipids of the chloroplast were extracted by the Bligh & Dyer (1959) procedure. The chloroformic extract was transferred into another vial and bleached overnight under u.v. light (362 nm). After evaporation of the solvent, 10 ml of scintillation mixture was added and the radioactivity was measured.

The transfer activity of a fraction was equal to $(a-b)$, a and b being respectively the percentage of the initial ^3H and ^{14}C radioactivities of the liposomes recovered in the organelles. The percentage of ^{14}C label, due to a non-exchangeable tracer (cholesteryl [^{14}C]oleate), was assumed to indicate the extent of cross-contamination of the organelles by the liposomes. The transfer activity (as a percentage of ^3H label) was thus corrected for this cross-contamination.

Results and Discussion

Transfer involving mitochondria

When protein extracts from spinach were applied to a column of Sephadex G-75, a large amount of material containing proteins of relative molecular mass greater than 100 000 was eluted in the void volume (Fig. 1). The fractions from this peak, introduced into liposome/mitochondria mixtures, showed negligible transfer activities (2–3% of corrected ^3H label). They contained lipolytic activities since the total radioactivity recovered at the end of the incubation decreased (results not shown).

By contrast, fractions of low relative molecular

Fig. 1. Gel filtration of phosphatidylcholine-transfer proteins from spinach cytosol on Sephadex G-75

The transfer activity (●) was determined by liposome/mitochondria assays. Liposomes contained [^3H]phosphatidylcholine ($2.5 \times 10^4 \text{ d.p.m.}; 350 \text{ nmol/ml}$) and cholesteryl [^{14}C]oleate ($2.0 \times 10^4 \text{ d.p.m./ml}$). Liposomes (0.5 ml) were incubated with mitochondria (2 mg of protein) in the presence of 3 ml of each fraction. 16-ml fractions were collected at a flow rate of 80 ml/h. The other experimental conditions are described in the text. The absorbance (—) was determined at 280 nm.

mass have a high transfer ability. With these fractions, more than 25% of the corrected ^3H label was recovered in the mitochondrial pellets after incubation, whereas only 1–2% of label was found in the absence of soluble protein. With these fractions the extent of cross-contamination was as low as that found without added protein (2–3% of ^{14}C label). The active fractions appeared as a broad peak with an elution volume similar to that observed for phosphatidylcholine-transfer protein from maize. This protein has a relative molecular mass of 20 000 (D. Douady, unpublished work). In our experiments, spinach proteins were assayed with liposomes and potato tuber mitochondria, since in previous investigations we have shown that the origin of the organelles has no major influence on the activity of the transfer proteins (Kader, 1975). Spinach cytosolic proteins were active with mitochondria prepared from spinach leaves (Julienne *et al.*, 1980) or pea seedlings (Julienne & Kader, 1981).

The active fractions from the column (more than 10% of ^3H label determined with 3 ml of the fractions) were pooled and concentrated with poly(ethylene glycol) or with an ultrafiltration cell (Amicon, PM10 membrane). When increasing amounts of the pooled fractions were introduced into liposome/mitochondria mixtures, the ^3H label increased in the mitochondrial pellets, whereas the ^{14}C label remained stable (Fig. 2). The $^3\text{H}/^{14}\text{C}$ ratio calculated from this experiment increased from 1.0 (in the absence of soluble protein) to 7.0 (in the presence of 3 mg of protein). Up to 30% of [^3H]phosphatidylcholine initially present in liposomes was transferred into mitochondria in the

presence of 3 mg of soluble protein, whereas only 3% of ^3H label was transferred in the presence of Tris/EDTA buffer. The activity of these fractions only slightly decreased within 2 weeks at $+4^\circ\text{C}$. However, 70% of the transfer ability was suppressed by a treatment of the soluble protein at 70°C for 30 min (Fig. 2).

The transfer process is time-dependent. No transfer was observed when cold solutions of liposomes, mitochondria and soluble proteins were mixed and immediately submitted to centrifugation. But when the mixture was incubated at 30°C for various times, the ^3H label increased, whereas the ^{14}C label remained constant in the mitochondrial pellets up to 60 min (Fig. 3).

Transfer involving chloroplasts

When the pooled fractions from the Sephadex column were introduced into mixtures comprising spinach chloroplasts and liposomes, $[\text{}^3\text{H}]$ phosphatidylcholine was recovered into chloroplasts after incubation (Fig. 4). Again, the extent of transfer of $[\text{}^3\text{H}]$ phosphatidylcholine toward

Fig. 3. *Transfer of phosphatidylcholine from liposomes to mitochondria mediated by spinach proteins*

Liposomes contained $[\text{}^3\text{H}]$ phosphatidylcholine (1.5×10^4 d.p.m.; 350 nmol/ml) and cholesteryl $[1\text{-}^{14}\text{C}]$ oleate (2.0×10^4 d.p.m./ml). Liposomes (0.5 ml) were incubated with mitochondria (2 mg of protein) in the absence of protein (○) or in the presence of 3 mg of soluble proteins (●) for various times of incubation. The ^3H radioactivity of the mitochondrial pellets is expressed in d.p.m.

Fig. 2. *Transfer of phosphatidylcholine from liposomes toward mitochondria mediated by spinach proteins*

Liposomes contained $[\text{}^3\text{H}]$ phosphatidylcholine (2.8×10^4 d.p.m.; 350 nmol/ml) and cholesteryl $[1\text{-}^{14}\text{C}]$ oleate (2.0×10^4 d.p.m./ml). Liposomes (0.5 ml) were incubated with mitochondria (2 mg of protein) in the presence of various amounts of soluble proteins (pooled fractions from the Sephadex G-75 column). The ^3H (●) and ^{14}C (○) radioactivities recovered in the mitochondrial pellets are expressed in d.p.m. The data obtained with assays performed with heat-treated proteins (70°C , 30 min) are indicated by arrows.

Fig. 4. *Transfer of phosphatidylcholine from liposomes to chloroplasts mediated by spinach proteins*

The experimental conditions were identical with those described in the legend to Fig. 2, except that chloroplasts were used instead of mitochondria.

chloroplasts depended on the amount of soluble proteins.

However, lower values than those found with mitochondria (10–15% instead of 25% of transfer) were obtained. The transfer activity was totally suppressed by the heat treatment (Fig. 4). The soluble proteins from spinach were also active with chloroplasts isolated from pea leaves (results not shown).

In conclusion, this work definitively establishes the presence of phosphatidylcholine transfer proteins in spinach leaves. The isolation procedure, which is the first to be performed on a photosynthetic tissue, involves a gel-filtration step that eliminates contaminating compounds. Fractions with a stable activity are thus obtained. These observations extend previous findings on the distribution of phospholipid-transfer proteins [previously named phospholipid-exchange proteins, since a reverse flow of phospholipids can also be detected (Wirtz, 1974; Zilversmit & Hughes, 1976; Kader, 1977)] to include photosynthetic tissue. Spinach phosphatidylcholine-transfer proteins can be very useful for examining the possible involvement of these proteins in the biogenesis of phospholipids and polyunsaturated fatty acids in chloroplasts as discussed by several authors (Julienne *et al.*, 1980; Yamada *et al.*, 1980; Roughan *et al.*, 1980; Jones & Harwood, 1980; Joyard *et al.*, 1980; Trémolières *et al.*, 1980; Julienne & Kader, 1981).

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