Phytochemistry, Vol. 24, No. 6, pp. 1163–1168, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

STIMULATION OF POTATO MICROSOMAL NADH–CYTOCHROME *c* REDUCTASE BY ACIDIC PHOSPHOLIPIDS*

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(*Received* 7 *June* 1984)

Key Word Index—*Solanum tuberosum*; Solanaceae; microsomes; electron transfers; phosphatidylserine; lipid-dependence.

Abstract—Potato microsomal membranes were solubilized by 0.5% sodium cholate solutions. Separation of lipids from proteins was realized by two successive gel filtrations on two different Sephadex columns. Lipid-free microsomal proteins maintained a high NADH–ferricyanide reductase activity but had a lowered (20%) NADH–cytochrome *c* reductase activity. The latter activity was strongly stimulated when lipid-free proteins were integrated, by sonication, into phosphatidylserine or phosphatidylinositol liposomes. Some stimulation was obtained also with phosphatidylcholine–lysophosphatidylcholine (7:3) mixtures. Other phospholipids were far less active or even inhibitory. Acidic phospholipids stimulate NADH–cytochrome *c* reductase activity by increasing noticeably the apparent affinities of enzymatic proteins for NADH or cytochrome *c*.

INTRODUCTION

The regulation of membrane-bound enzyme activities by the phospholipids which surround the proteins within the membranes is well established in animal tissues [1, 2]. Some enzymes exhibit a certain specificity in their lipid requirement for optimal activity. Thus, beef heart β-hydroxy-butyrate dehydrogenase requires phosphatidylcholine [3]; liver glutaminase requires phosphatidylcholine or phsophatidylethanolamine [4]; rabbit kidney $(Na^+ + K^+)$-dependent ATPase requires phosphatidylserine [5] and phosphatidylserine is the sole phospholipid effective for the activation of Ca^{2+} activated, phospholipid-dependent protein kinase from rat brain [6]. By contrast, the lipid-dependence of membranous enzymes from plant tissues is less clearly established [7, 8]. The question of the specificity of the lipids required for optimal activity is entirely open in the case of plant enzymes. In this paper we show that NADH–cytochrome *c* reductase from potato microsomes requires acidic phospholipids (phosphatidylserine or phosphatidylinositol) for optimal activity; these phospholipids modify the apparent affinities of enzymatic proteins for their substrates (NADH or cytochrome *c*).

RESULTS

Solubilization and delipidation of microsomal proteins

Potato microsomal membranes were solubilized by a 0.5% cholate solution, as indicated in the Experimental.

Figure 1 shows that, following gel-filtration of a Sephadex G 150 column, the separation of membrane constituents was excellent: most membrane proteins, previously labelled with ^{3}H-leucine, were effectively eluted with the void-volume (fractions 15–30); the proteins involved in electron-transport systems

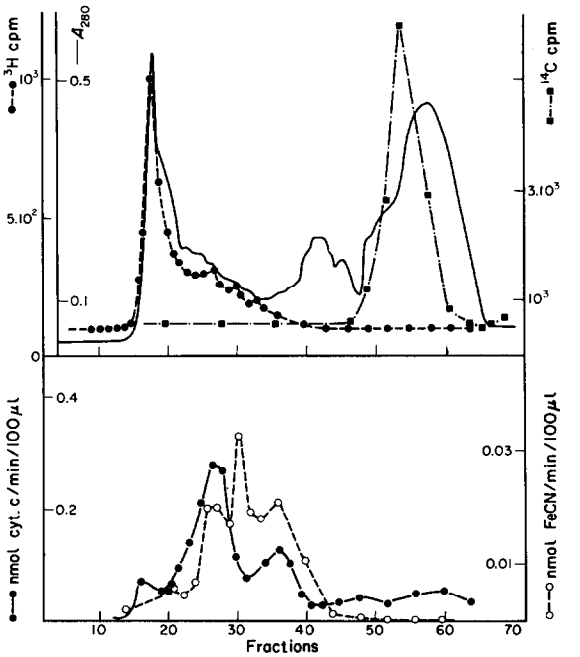

Fig. 1. Separation of membrane lipids and proteins by gel-filtration on a Sephadex G 150 column. Microsomal lipids and proteins were labelled by ^{14}C-ethanolamine and ^{3}H-leucine respectively. Microsomes were solubilized by a 0.5% cholate solution (see text for experimental details). Upper graph: ——, *A* 280 nm; ■—·—■, ^{14}C; ●– – –●, ^{3}H. Lower graph: ●—●, NADH-cytochrome *c*; ○—○, NADH–ferricyanide activities.

*Part 4 in the series "Regulation by Lipids of Plant Microsomal Enzymes".

Table 1. Variations in NADH–ferricyanide and NADH–cytochrome *c* reductase activities following lipid-depletion of membranes proteins or reconstitution of artificial membrane with phospholipid liposomes (results of a typical experiment)

	NADH–cyt. *c.* reductase (nmol cyt. *c.* reduced/min/mg protein)	NADH–ferricyanide reductase (nmol ferricyanide reduced/min/mg protein)
Microsomal membranes	48.8	268
Solubilized membranes (in presence of cholate)	24.6	410
Delipidated proteins (in presence of cholate)	8.6	—
Delipidated proteins (in absence of cholate, after freezing and thawing)	13.3	431
Reconstituted membranes (with PS liposomes)	92.5	—

(NADH–ferricyanide reductase and NADH–cytochrome *c* reductase) were slightly included in the Sephadex gel and eluted with fractions 20 to 40 (i.e. $V_e = 2V_v$); membrane lipids (labelled with ^{14}C-ethanolamine) were eluted with fractions 50 to 60 (i.e. $V_e = 3V_v$). The lipid-dependent NADH–cytochrome *c* reductase activity was considerably decreased after this delipidation treatment (Table 1) whereas NADH–ferricyanide activity, which increased per mg of protein, appeared as a non-lipid-dependent activity.

The lipid-depleted proteins eluted from the Sephadex G 150 column were maintained in solution by sodium cholate. This detergent could be eliminated by gel-filtration on a Sephadex G 25 column, as recommended by Rogers and Strittmatter [9]: proteins freed from detergent were eluted with the void volume of this second column whereas cholate, included in Sephadex, was eluted far later. Membrane proteins, obtained in this manner, were delipidated and decholated; they aggregated in aqueous solutions. NADH–cytochrome *c* reductase activity associated with these proteins was 75% lower whereas NADH–ferricyanide activity was 60% higher than the corresponding activities of starting microsomes (Table 1). It is concluded that delipidation impaired the functioning of NADH–cytochrome *c* reductase, a lipid-dependent enzyme complex, while NADH–ferricyanide reductase would be a non lipid-dependent protein, as already found previously [7]. Membrane proteins, thus delipidated and decholated, were used for reconstitution experiments.

Membrane reconstitutions

The lipid-depleted proteins were included by sonication into various types of phospholipid liposomes, as described in the Experimental. Figure 2 shows, for example, the results of a typical experiment, obtained after inclusion of microsomal proteins into two different types of liposomes made of either pure phosphatidylcholine (PC) or a mixture of phosphatidylethanolamine (PE) and phosphatidylcholine (PE:PC, 3:1 w/w). The NADH–cytochrome *c* reductase activity of the delipidated proteins was lowered when they were included in pure PC liposomes; by contrast, membrane reconstitutions with the PE:PC mixture increased the enzyme activity of the proteins.

This experiment showed clearly that different lipid environments have different effects on NADH–cytochrome *c* reductase activity. The best stimulations of NADH–cytochrome *c* reductase activity were obtained when microsomal proteins were included into acidic phospholipid liposomes (Figs 3 and 4). Thus phosphatidylserine (PS) or phosphophatidylinositol (PI) were very effective in restoring the NADH–cytochrome *c* reductase activity of delipidated microsomal proteins. However, when proteins were included in PS liposomes, a rapid cytochrome *c* reduction occurred during the first 30 sec and then a plateau was reached. Expressed as per mg of protein, the enzyme activity in membranes reconstituted with PS liposomes was about twice (Table 1) that in the starting microsomal membranes. This can be explained by the relative purification of enzymatic proteins during gel-filtration on the Sephadex G 150 column.

As a previous study by Jones and Wakil [10] on hen liver microsomes, delipidated by 10% aqueous acetone treatments, seemed to indicate that mixtures of phosphatidylcholine and lysophosphatidylcholine (7:3 w/w) were the most effective in restoring NADH–cytochrome *c* reductase activity, we did some membrane reconstitution experiments with these phospholipids. Figure 5 shows that effectively, enzyme activity stimulations were obtained with mixtures of phosphatidylcholine (PC) and lysophosphatidylcholine (lyso PC). In our hands, the best stimulations of initial velocity were obtained with a mixture having the following proportions: PC/lyso PC (8:2 w/w). However the enzyme appeared to be very quickly inhibited by this particular mixture: a plateau was reached after 1 min (followed by an apparent reoxidation of cytochrome *c*, a phenomenon which remains unexplained at the moment). On the other hand, the mixture found to be the most effective by Jones and Wakil (i.e. PC:lyso PC in the proportions 7:3) resulted in an enzyme activity stimulation which remained stable for several minutes. It is important to compare the enzyme stimulations obtained in the experiments reported in Figs 3 and

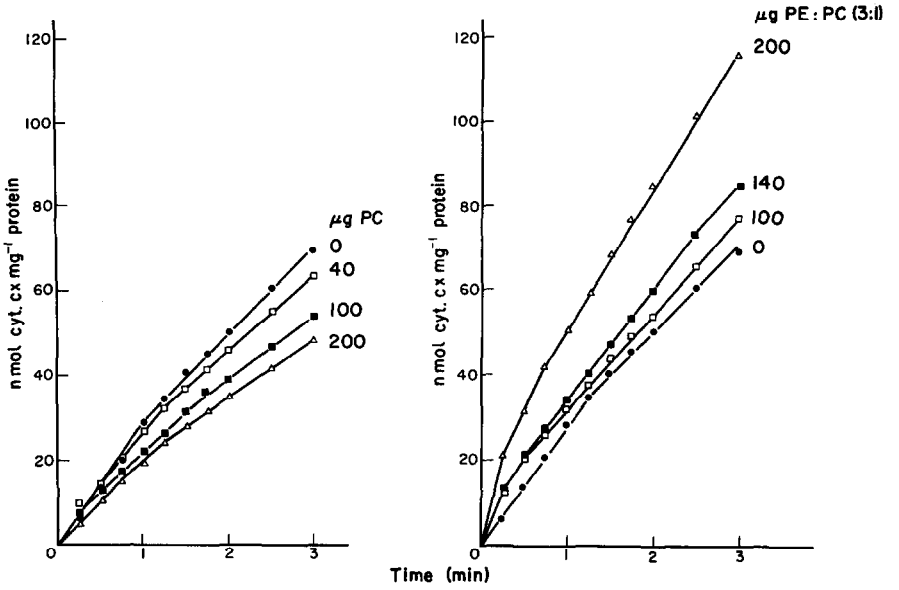

Fig. 2. Effects of membrane reconstitutions on NADH–cytochrome *c* reductase activity. Potato microsomal proteins, delipidated and decholated by gel-filtration (see Experimental) were included by 10 sec sonication either into pure phosphatidylcholine liposomes (left) or into PC:PE (1:3) liposomes (right). The enzyme activities were measured spectrophotometrically, by following the reduction of cytochrome *c* by the increase in absorption at 550 nm.

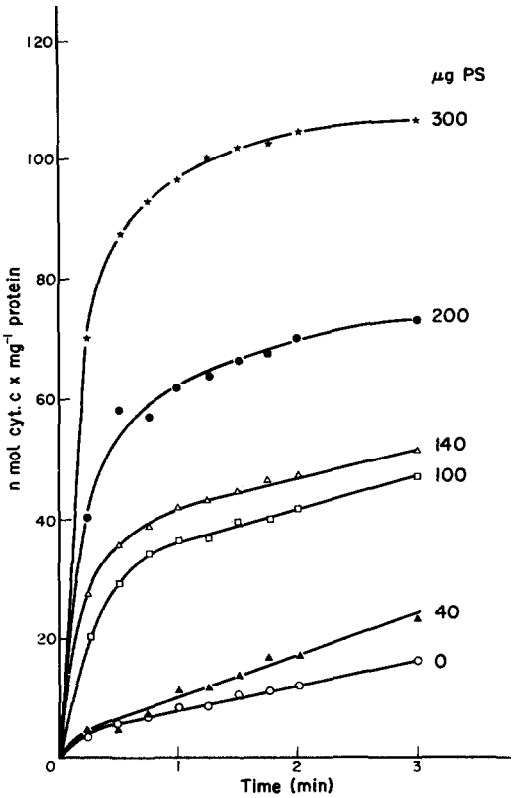

Fig. 3. Effects of membrane reconstitutions with increasing quantities of pure PS liposomes on NADH–cytochrome *c* reductase activity. The enzyme activities are measured spectrophotometrically, by following the reduction of cytochrome *c* by the increase in absorption at 550 nm. 100 μg of delipidated proteins were used in each assay.

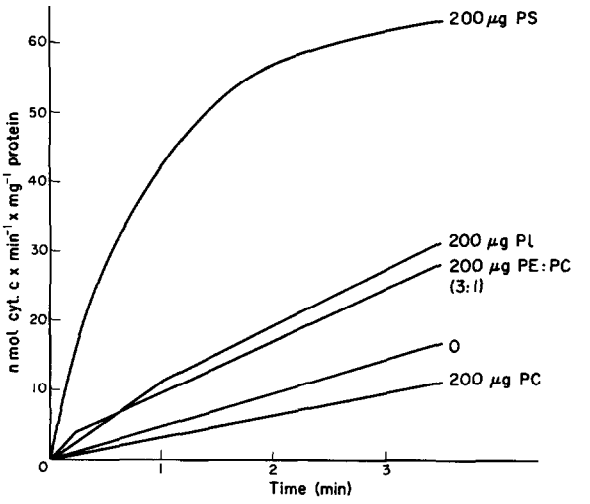

Fig. 4. Effects of the inclusion of delipidated microsomal proteins into various types of phospholipid liposomes on NADH–cytochrome *c* reductase activity. Two hundred μg of the various molecular species of phospholipid, in the form of liposomes, were sonicated for 10 sec with 100 μg of delipidated proteins, as indicated in Experimental. NADH–cytochrome *c* reductase activity was followed by the increase in absorption at 550 nm due to the reduction of cytochrome *c*.

5: it is clear that membrane reconstitutions done with pure acidic phospholipids (PS or PI) caused far higher NADH–cytochrome *c* reductase stimulations than membrane reconstitutions done with the mixtures of PC and lyso PC. For instance the highest initial velocities obtained with pure PS liposomes (100 nmol of cytochrome *c*

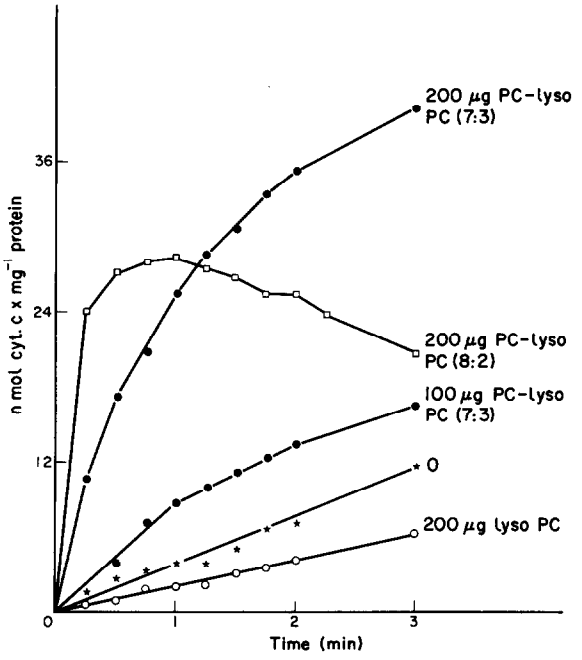

Fig. 5. Effects of membrane reconstitutions with various mixtures of phosphatidylcholine (PC) and lysophosphatidylcholine (lyso PC) on NADH–cytochrome *c* reductase activity. The quantities of lipids added to 100 μg of delipidated proteins are indicated in the right part of the graph. NADH–cyt. *c* reductase activity was measured spectrophotometrically as in Figures 2–4.

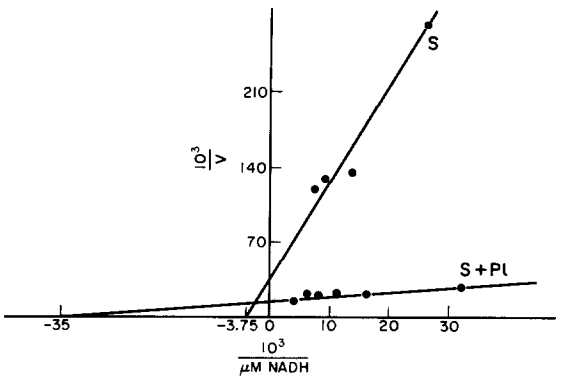

Fig. 6. Lineweaver and Burk plot of NADH–cytochrome *c* reductase activity measured as a function of NADH concentration. S, standard, lipid-free microsomal proteins; S + PI, microsomal proteins included in PI liposomes. V = nmoles of cytochrome *c* reduced/min/mg protein.

reduced/mg protein/min, Fig. 3) were five times greater than the best activities obtained with PC–lyso PC mixtures (20 nmol of cytochrome *c* reduced/mg protein/min, Fig. 5).

Effects of activating phospholipids on enzyme affinities

To see whether delipidated and relipidated proteins had the same or different affinities for NADH and cytochrome *c*, experiments were done, in which the quantities of substrates given to the enzymes in the cuvettes of the spectrophotometer were varied. The results of such an experiment are given in Fig. 6. It can be seen that delipidated microsomal proteins and microsomal proteins included in PI liposomes have very different affinities for NADH: apparent K_m 266 and 28.6 μM respectively for delipidated and relipidated proteins. We conclude that inclusion of the microsomal proteins of the NADH–cytochrome *c* reductase system in an acidic phospholipid environment increases strikingly the apparent affinity of the enzymes for NADH. A similar conclusion can be reached when varying cytochrome *c* concentrations: in that case too, microsomal proteins included in PI liposomes had far greater affinities for cytochrome *c* (apparent K_m 11.8 μM) as compared with delipidated microsomal proteins (apparent K_m 250 μM).

DISCUSSION

Insensitiveness of microsomal NADH–ferricyanide reductase activity to lipid environment

As already suggested in earlier studies [7], the microsomal flavoprotein transferring electrons from NADH to ferricyanide is easily detached from membranes by cholate; the enzyme appears to be some 60% more active in the solubilized form as compared with the membrane-bound enzyme (Table 1). This increase in activity can be explained by the relative enrichment in active proteins obtained in the fractions collected after gel-filtration on the Sephadex G 150 column (Fig. 1). These results show that microsomal NADH–ferricyanide reductase does not require any lipid environment to function.

Lipid-dependence of microsomal NADH–cytochrome c reductase activity

In a previous work [7], the phospholipid dependence of microsomal NADH–cytochrome *c* reductase was suspected as a result of the delipidation of microsomal membranes by phospholipase C: removal of phospholipids from the membranes was correlated with loss of enzyme activity and some restoration (11%) of that activity could be obtained by adding back the extracted phospholipids to lipid-depleted membranes. However, when microsomal fractions were treated with phospholipases, the resulting inactivations of the enzymes might have been due to accumulation of phospholipid degradation products rather than phospholipid destruction itself. Therefore it was highly desirable, in order to prove the lipid-dependence of NADH–cytochrome *c* reductase, to achieve the delipidation of membrane proteins by a method not involving phospholipid hydrolysis. This step was realized in the present work by means of a double gel-filtration on two different Sephadex columns. This technique allowed us to obtain a heterogeneous mixture of lipid-free microsomal proteins, aggregated in water, probably by hydrophobic interactions. Membrane reconstitutions were obtained by sonicating these proteins with phospholipid liposomes: phospholipid and sonication apparently allowed reconstitution by dispersing the aggregates so that the hydrophobic portions of the microsomal proteins could interact with liposomes. In certain types of reconstituted membranes, the NADH–cytochrome *c* reductase activity was much higher than the activity in aggregated lipid-free proteins. We thus

proved the so-called 'lipid-dependence' of microsomal NADH–cytochrome *c* reductase. The requirement for lipids of this enzyme had been already found by Jones and Wakil [10] using hen microsomes and by Rogers and Strittmatter [9] using rabbit liver microsomes. These authors had used different methods; Jones and Wakil delipidated microsomes with aqueous solutions and Rogers and Strittmatter added sodium deoxycholate to the total mixture of lipids extracted from the microsomes to obtain membrane reconstitution. In our work, we tried to incorporate directly lipid-free proteins into pure phospholipid liposomes in order to be able to test the specific effects of the various phospholipids upon enzyme activity.

Specificity of the different phospholipids for NADH–cytochrome c activity stimulation

Only acidic phospholipids (i.e. phosphatidylserine, phosphatidylinositol or a mixture of phosphatidylcholine and lysophosphatidylcholine) allowed high NADH–cytochrome *c* reductase activities after integration of lipid-free proteins into liposomes. Several membrane proteins have already been shown to bind acidic lipids preferentially, using differential scanning calorimetry or spin labelled lipids. The myelin proteolipid lipophilin binds phosphatidylserine, phosphatidic acid or phosphatidylglycerol preferentially over phosphatidylcholine [11]. Rhodopsin binds phosphatidylserine preferentially to phosphatidylglycerol or phosphatidic acid compared to phosphatidylcholine [12] while (Na^+, K^+)-ATPase binds negatively charged lipids preferentially over neutral or positively charged lipids [13]. The stimulation of NADH–cytochrome *c* reductase activity brought about by acidic phospholipids, as demonstrated in the present work, also suggests preferential binding of these lipids to enzymic proteins, in a manner which could affect their active sites, since the apparent affinities of the proteins for NADH or cytochrome *c* were greatly increased by acidic phospholipids.

Acidic phospholipids do not always stimulate membrane-bound enzyme activities: for instance, an inactivated solubilized preparation of adenylate cyclase could not be activated by phosphatidylserine, phosphatidylinositol or phosphatidic acid but instead, these acidic phospholipids inhibited the normal stimulation of enzyme activity by phosphatidylcholine [14].

Concerning the specific requirements of acidic phospholipids for NADH–cytochrome *c* reductase stimulation, we have made one intriguing observation: in some preparations lipid-free membrane proteins could be stimulated more actively by phosphatidylserine than by phosphatidylinositol whereas in other preparations, phosphatidylinositol was the best activator. Both types of phospholipids are acidic but the reason why one type of negatively charged polar head is preferred over the other is unclear at the moment since the lipid-requirement for optimal activity changes from batch to batch of lipid-depleted proteins. It is presumably difficult to always remove the last traces of detergent during the preparation of proteins and this will undoubtedly affect the reconstitution. To determine more precisely the specific lipid requirements of the proteins (flavoprotein and cytochrome *b*) constituting the NADH–cytochrome *c* reductase system, it will be necessary to extract and purify these proteins from microsomal membranes and then to integrate them into various types of phospholipid liposomes.

EXPERIMENTAL

Potatoes were furnished by the "Institut technique de la Pomme de terre" (St Rémy Lhonoré, France).

Labelling of lipids and proteins. 25 μCi of ^{3}H-leucine (50 Ci/mmol) or ^{14}C-éthanolamine (50 mCi/mmol) solutions were deposited as microdroplets (total vol 200 μl) upon aging slices of potato tubers. To perform the aging treatment, tuber slices (1 mm thick) were shaken (to oxygenate the soln) over a 0.1 mM $CaSO_4$ solution, at 25°, for 16 hr [15].

Preparation of microsomes. 160 g of potato tuber (fresh parenchyma or labelled slices) were ground in a roller mill [16]. The grinding medium was 0.4 M sucrose, 1 mM $MgCl_2$, 10 mM KCl, 0.75% bovine serum-albumin, 4 mM cysteine hydrochloride in 0.1 M Tris–HCl buffer, pH 8. The ratio material/medium was about 1:1 (w/v). After filtration through Miracloth and cheese-cloth, the homogenate was centrifuged at 12 500 *g* for 20 min. The supernatant was further centrifuged at 100 000 *g* for 60 min. After discarding the supernatant, the resulting pellet constituted the microsomal membranes used for solubilization.

Solubilization of microsomal membranes. Microsomal membranes (final concentration: 10 mg of protein/ml) were stirred at 0° for 15 min in a medium (pH 8.0) containing sodium cholate (final concentration: 0.5%), 25 mM Tris–HCl and 127 mM KCl. After dilution with an equal vol of buffer, insolubilized material (about 50% of initial proteins) was removed by centrifugation at 148 000 *g* for 2 hr.

Separation of lipids and proteins was carried out by a method first proposed by Graham *et al.* [17]. 5 ml of the supernatant fraction (containing about 25 mg of protein) were applied to a column (80 cm × 2.5 cm; void volume 155–160 ml) of Sephadex G 150 equilibrated with a soln (pH 8.0) containing 0.25% cholate, 25 mM Tris–HCl and 127 mM KCl. The column was eluted at a flow rate of 40 ml/hr with the same soln and 8 ml fractions were collected. All operations were carried out at approximately 4°.

Separation of membranous proteins from cholate was achieved following Rogers and Strittmatter [9]. Microsomal proteins eluted from the Sephadex G 150 column were concentrated by dialysis against solid polyethyleneglycol and cholate was removed by gel filtration on a Sephadex G 25 column equilibrated with a soln of 25 mM Tris–HCl buffer (pH 8), 0.2 mM Na, EDTA acid, 0.1 M NaCl. Delipidated and decholated proteins were eluted with the same soln at a flow rate of 80 ml/hr; proteins were collected in the void vol of this last column and concentrated as indicated above. The lipid-depleted proteins were immediately used for reconstitution experiments or frozen in liquid N_2 and utilized within one month.

Measurement of NADH–ferricyanide and NADH–cytochrome c reductase activities. NADH–ferricyanide reductase activity was measured following Lee *et al.* [18] and NADH–cytochrome *c* reductase, following Hackett *et al.* [19], except that the pH was fixed at 8.0, as recommended by Moreau [20] and Philipp *et al.* [21]. Enzymatic activities were determined spectrophotometrically, at 25°. Reference and assay cuvettes contained the same mixtures (NADH, ferricyanide or cytochrome *c*, Tris–HCl buffer, pH 8.0, and phospholipid liposomes when indicated) except for microsomal proteins which were omitted from the reference mixture. The enzymatic reactions were initiated by the addition of NADH. The presence of liposomes in both the reference and the assay cuvettes was absolutely required to eliminate the absorption artefacts resulting from binding of ferricytochrome *c* to phospholipid vesicles [22]. With our spectrophotomer

(Shimadzu UV 240) absorption data could be collected only every 15 sec.

Reconstitution of artificial membranes. Phospholipids used for reconstitution experiments were of commercial origin (Sigma, St. Louis, Missouri, U.S.A.). The purity of commercial preparations was checked by TLC. 'Liposomes' (mostly unilamellar vesicles) were prepared by sonication [8]. 2 mg of phospholipids were deposited under 1 ml 10 mM Tris–HCl buffer (pH 8) and sonicated for 20 min, under N_2, in a M.S.E. apparatus, at the highest power and at 20° (damage to phospholipids due to their exposure to ultrasound could not be controlled and could vary from preparation to preparation). 100 μl of liposome suspension (200 μg of phospholipid) were added to 100 μg membrane protein dissolved in Tris–HCl buffer (final volume: 200 μl) in a 2 ml conical plastic tube and the mixture was sonicated at the highest power of the M.S.E. apparatus, at 20° and under N_2, for 10 sec. This treatment allowed the membrane proteins to be integrated into phospholipid liposomes. These artificial membranes were immediately utilized for measurement of enzymatic activities.

Protein contents were determined according to Lowry *et al.* [23].

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