Translocation to rat liver mitochondria of phosphatidate phosphohydrolase

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When a particle-free supernatant fraction from rat liver was incubated at 37 °C with mitochondria and oleate, some of the enzyme phosphatidate phosphohydrolase (PAP), initially present in the particle-free supernatant, was recovered, after the incubation, bound to mitochondria. This translocation of PAP from cytosol to mitochondria was stimulated by oleate or palmitate in a similar fashion to the stimulation of translocation of PAP to endoplasmic reticulum [Martin-Sanz, Hopewell & Brindley (1984) FEBS Lett. 175, 284–288]. Translocation of PAP from particle-free supernatant to a partially purified mitochondrial-outer-membrane preparation was also stimulated by oleate. More PAP was bound to a mitochondrial-outer-membrane fraction washed in 0.5 M-NaCl before resuspension in sucrose than to a sucrose-washed mitochondrial-outer-membrane preparation. In contrast, washing of microsomal membranes in 0.5 M-NaCl did not enhance the binding of PAP to these membranes. PAP also binds to phosphatidate-loaded mitochondria or microsomes (microsomal fractions). In the experimental system employed, more PAP bound to mitochondria loaded with phosphatidate than to microsomes loaded with phosphatidate. The results are discussed in relation to the role of mitochondrial phosphatidate in liver lipid metabolism.

INTRODUCTION

Phosphatidate phosphohydrolase (PAP) is an ambiquitous enzyme. Thus it occurs both in the cytoplasm and attached to cell membranes [1,2], and its distribution between cell compartments can be modified [1–3]. Regulation of the intracellular movement or translocation of PAP appears to be important in regulating the rate of synthesis of triacylglycerols [4].

Translocation of cytosolic PAP to endoplasmic reticulum has been demonstrated in cell-free systems prepared from rat liver [5] or adipose tissue [6]. Translocation to mitochondria of adipose tissue has also been shown *in vitro* [6]. Experiments, described below, were carried out to determine whether translocation also occurs to mitochondria of rat liver.

Phosphatidate, the substrate for PAP, is synthesized both in endoplasmic reticulum and in mitochondria in rat liver. The capacity of mitochondria to synthesize phosphatidate *in vitro* was observed to be about one-third of total homogenate activity [7]. The contribution of mitochondria to total phosphatidate synthesis *in vivo* is not known.

Mitochondrial phosphatidate formation occurs in the outer membrane [7,8]. In this membrane there is an active glycerophosphate acyltransferase [3,7–9] which is susceptible to proteolytic inactivation on both the cytoplasmic [10] and inner surface [10,11], suggesting it is a transmembrane protein. These studies do not identify the location of the active site of glycerophosphate acyltransferase. The product of glycerophosphate acyltransferase activity is lysophosphatidate, which is converted into phosphatidate by monoacylglycerophosphate acyltransferase. The location of the latter

enzyme in the outer membrane is not known. Thus the actual site of formation of phosphatidate is not known either. If it is formed in the outer surface of the membrane, it could be a substrate for the cytoplasmic PAP. Mitochondrial phosphatidate was shown to be a substrate for cytosolic PAP *in vitro* [12]. Provided that the mitochondrial membranes were unbroken, this suggests that phosphatidate is accessible in the outer surface. To investigate this hypothesis, the translocation of PAP to rat liver mitochondrial outer membrane was studied.

The translocation of PAP to mitochondria and microsomes (microsomal fractions) that had been preloaded with phosphatidate was also investigated. The results are discussed in relation to the role of mitochondrial phosphatidate in liver lipid metabolism.

EXPERIMENTAL

Materials

Male Wistar rats (180–200 g) were used. The animals were fed on diet 41B, from E. Dixon and Sons, Ware, Herts., U.K. This contained (w/w) 46.7% carbohydrate (mainly starch), 15.4% protein, 3.1% fat and 4.4% crude fibre. Food and water were available *ad libitum*. [^{14}C]Oleic acid (50 Ci/mol) and [^{14}C]tyramine hydrochloride (50 Ci/mol) were purchased from Amersham International, Amersham, Bucks., U.K. ADP and sucrose were from BDH, Poole, Dorset, U.K. Other chemicals were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Solvents used were of A.R. grade. The sources of aluminium oxide [13] and t.l.c. plates [14] were described in the references.

Abbreviation used: PAP, phosphatidate phosphohydrolase (EC 3.1.3.4).

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Preparation of mitochondrial, microsomal and particle-free-supernatant fractions for translocation experiments *in vitro*

The fractions were prepared as described [7], except that livers were homogenized in approx. 5 ml of 0.88 M-sucrose/2 mM-EDTA/5 mM-Tris/HCl (pH 7.4) per g instead of 7.5 ml per g. A purified mitochondrial fraction was prepared by density-gradient centrifugation [7], diluted with 0.88 M-sucrose/2 mM-EDTA/5 mM-Tris/HCl, pH 7.4, and centrifuged at 12000 *g* ($r_{av.} = 5.2$ cm) for 15 min. The pellet was resuspended in the same sucrose solution at a concentration of approx. 25 mg of protein/ml.

In some experiments, a combined mitochondria-free supernatant fraction was centrifuged at 110000 *g* ($r_{av.} = 7.8$ cm) for 120 min at 4 °C. The supernatant was retained as the particle-free supernatant. In other experiments, in which a microsomal fraction was required, the mitochondria-free supernatant fraction was centrifuged at 60000 *g* ($r_{av.} = 7.8$ cm) for 20 min at 4 °C. The resulting supernatant was then further centrifuged at 110000 *g* ($r_{av.} = 7.8$ cm) for 120 min at 4 °C. The microsomal pellet was resuspended in 0.25 M-sucrose/20 mM-Hepes, pH 7.4.

Particle-free supernatant and mitochondrial fractions were diluted 3.5-fold with nano-pure water, by adding the latter dropwise and mixing gently, to give a final concentration of 0.25 M-sucrose. Hepes buffer (1 M, pH 7.4) was added to give a final concentration of 20 mM. The dilution was carried out immediately before the translocation experiment described below.

Preparation of partially purified mitochondrial outer membrane

A mitochondrial fraction was prepared from four rat livers as described above. A mitochondrial-outer-membrane fraction was prepared from this as described by Martinez & McCauley [15].

The band at the interface between 0.45 M-sucrose and 1.2 M-sucrose was collected. This fraction was divided into two equal portions: one was mixed with an equal volume of 0.25 M-sucrose/20 mM-Hepes, pH 7.4, and the other mixed with an equal volume of 1 M-NaCl. After centrifugation for 40 min at 200000 *g* ($r_{av.} = 6.4$ cm) at 4 °C, the pellets were each resuspended in 1 ml of 0.25 M-sucrose/20 mM-Hepes, pH 7.4.

Preparation of phosphatidate-loaded mitochondria or microsomes

(i) **Phosphatidate-loaded mitochondria.** The method was based on that of Smith *et al.* [12]. A 10 ml cocktail was prepared on ice containing, in order of addition: 22 mM-sodium phosphate buffer, pH 7.4, 50 mg of bovine serum albumin, 6.6 mM-ATP, 6.6 mM-MgCl₂, 2.23 mM-GSH, 0.033 mM-CoA and 20 mM-*sn*-glycerol 3-phosphate. A volume of 0.88 M-sucrose was added so that the final concentration of sucrose, after addition of mitochondria, was 0.25 M. This cocktail was warmed to 37 °C for 5 min. Palmitate and [¹⁴C]oleate (0.1 Ci/mol), prepared by warming the acids with a 16% molar excess of KOH, were added to the cocktail to final concentrations of 0.4 mM and 0.3 mM respectively. A further 5 min incubation at 37 °C preceded the addition of 2 ml of mitochondrial fraction which had been warmed to 37 °C for 1 min before addition. The resulting mixture

was incubated for 60 min at 37 °C, cooled to 0 °C, layered over 20 ml of 0.88 M-sucrose/2 mM-EDTA/5 mM-Tris/HCl, pH 7.4, and centrifuged at 12000 *g* ($r_{av.} = 5.2$ cm) for 15 min at 4 °C. The pellet was suspended in 1 ml of 0.88 M-sucrose/2 mM-EDTA/5 mM-Tris/HCl, pH 7.4.

(ii) **Phosphatidate-loaded microsomes.** A 10 ml cocktail was prepared on ice containing 22 mM-sodium phosphate buffer, pH 7.4, 50 mg of bovine serum albumin, 8.0 mM-ATP, 13.4 mM-MgCl₂, 5 mM-dithiothreitol, 0.033 mM-CoA, 12 mM-*sn*-glycerol 3-phosphate and 10 mM-NaF. A volume of 0.88 M-sucrose was added so that the final concentration of sucrose, after addition of microsomes, was 0.25 M. This cocktail was warmed to 37 °C for 5 min. Palmitate and [¹⁴C]oleate (0.1 Ci/mol), warmed to 65 °C, were added to the cocktail to final concentrations of 0.4 mM and 0.3 mM respectively. A further 5 min incubation at 37 °C preceded the addition of 2 ml of microsomal fraction which had been warmed to 37 °C for 1 min before addition. After incubation for 60 min at 37 °C the microsomal suspension was cooled to 0 °C and layered in 2.5 ml portions over 6 ml of 0.44 M-sucrose. After centrifugation at 200000 *g* ($r_{av.} = 6.4$ cm) for 40 min at 4 °C, the microsomal pellets were combined and resuspended, by homogenization, in 1 ml of 0.88 M-sucrose/2 mM-EDTA/5 mM-Tris/HCl, pH 7.4.

Translocation experiments

The redistribution of PAP from particle-free supernatant to membranes was investigated in the following experiments. Membrane and supernatant fractions obtained after 'translocation' incubations were stored at -20 °C before enzyme assays were carried out.

(i) **Translocation of PAP to mitochondria.** Particle-free supernatant (1 ml) and mitochondrial fraction (0.4 ml) were preincubated together for 2 min at 37 °C. A further 10 min incubation followed the addition of oleate or palmitate. Palmitate and oleate solutions were prepared by warming the acids with a 16% molar excess of KOH. Final concentrations of fatty acid in the incubation were 0.37 mM or 0.75 mM. Controls containing no fatty acid and samples of particle-free supernatant alone were also incubated, with the samples described above. At the end of the incubation, samples were placed on ice. Centrifugation was carried out at 12000 *g* in a MSE MicroCentaur for 10 min at 4 °C. The mitochondrial pellets were resuspended gently in 0.2 ml of 0.25 M-sucrose/20 mM-Hepes, pH 7.4.

(ii) **Translocation of PAP to partially purified mitochondrial outer membrane and to microsomal membranes.** Mitochondrial-outer-membrane fraction or microsomes and sucrose (0.5 ml total) and particle-free supernatant (1.0 ml) were incubated in the absence or presence of oleate (0.37 mM) for 10 min at 37 °C. After cooling on ice, the samples were centrifuged at 200000 *g* ($r_{av.} = 6.4$ cm) for 45 min at 4 °C. The pellet was resuspended in 0.2 ml of 0.25 M-sucrose/20 mM-Hepes, pH 7.4. Before the translocation incubation, the microsomal fraction was washed either with sucrose or in 0.5 M-NaCl as described above for the mitochondrial-outer-membrane fraction.

(iii) **Translocation of PAP to phosphatidate-loaded mitochondria and phosphatidate-loaded microsomes.** Phosphatidate-loaded mitochondrial fraction, phosphatidate-loaded microsomal fraction and the corresponding mitochondrial and microsomal fractions from which the phosphatidate-loaded membranes were prepared, were freshly prepared in 0.88 M-sucrose/2 mM-EDTA/5 mM-Tris/HCl, pH 7.4, and were diluted 3.5-fold in nano-pure water as described above. Hepes (1 M, pH 7.4) was added to give a final concentration of 20 mM. Various combinations of mitochondrial and microsomal membrane fractions were incubated with particle-free supernatant and 10 mM-NaF for 10 min at 37 °C. After cooling samples to 0 °C, centrifugation was carried out at 12000 *g* for 10 min at 4 °C. The resulting supernatants were centrifuged at 200000 *g* ($r_{av.} = 6.4$ cm) for 45 min at 4 °C. The low-speed pellets and the high-speed pellets were resuspended in 0.2 ml of 0.25 M-sucrose/20 mM-Hepes, pH 7.4.

Enzyme and protein determinations

PAP [13], succinate dehydrogenase [16], NADPH-cytochrome *c* reductase [17], rotenone-insensitive NADH-cytochrome *c* reductase [7] and monoamine (benzylamine) oxidase [7] were assayed as described in the references given.

Monoamine oxidase was also assayed by a modification of the method of Russell *et al.* [18]. Sample (0.05 ml) was preincubated for 2 min at 30 °C with 0.09 ml of 0.1 M-potassium phosphate buffer, pH 7.2. The reaction was started by the addition of 0.01 ml of 5 mM-[7-¹⁴C]tyramine hydrochloride (0.48 Ci/mol). After 30–40 min at 30 °C the reaction was stopped with 0.1 ml of ice-cold 2 M-HCl, and 3 ml of toluene/ethyl acetate (1:1, v/v) containing 1% 2,5-diphenyloxazole was added. The mixture was vigorously shaken, and each sample was vortex-mixed for 10 s and centrifuged at 1000 rev./min for 20 min at 4 °C. [7-¹⁴C]Tyramine hydrochloride radioactivity was determined after 12 h. The reaction rate was proportional to amount of enzyme protein. Each sample was assayed at three protein concentrations.

Protein was determined by the biuret method [16] or by that of Lowry *et al.* [19], with bovine serum albumin as standard.

Determination of phosphatidate

To 0.5 ml of phosphatidate-loaded membrane fraction was added 0.6 ml of water, 0.1 ml of 6 M-HCl and 4.5 ml of chloroform/methanol (1:2, v/v). The mixture was vortex-mixed and then left at room temperature for 30 min. To the mixture was added a further 1.5 ml of chloroform and 1.5 ml of 2 M-KCl in 0.2 M-H₃PO₄. After mixing and centrifuging at 2000 rev./min for 10 min, the top phase was washed with 2 × 5.7 ml of synthetic top phase and then dried under a stream of N₂. The dry lipid extract was dissolved in 0.125 ml of chloroform. Lipid extract (0.05 ml) was applied to glass-backed t.l.c. plates and lipid separation performed as described in ref. [19]. Areas containing phosphatidate were scraped from the plate and eluted by washing with 2 × 2 ml of chloroform/methanol (1:4, v/v). The solvents were evaporated under a stream of N₂, and lipid P was determined by the method of King [20].

RESULTS

Translocation of PAP to mitochondria *in vitro*

Evidence is presented in Table 1 that PAP is able to translocate from particle-free supernatant to mitochondria *in vitro* in the presence of added fatty acid. The addition of 0.37 mM-oleate, 0.75 mM-oleate or 0.75 mM-palmitate to the incubation produced a significant translocation of PAP activity from the particle-free supernatant to the mitochondrial fraction when compared with a control to which no fatty acid had been added. Palmitate was less effective than oleate in facilitating the translocation. At concentrations of oleate or palmitate above 0.8 mM the activity of PAP, in both particle-free supernatant and mitochondrial fraction, was inhibited (results not shown).

Translocation of PAP to mitochondrial outer membrane

Oleate stimulated a significant translocation of PAP activity from the particle-free supernatant to the mitochondrial-outer-membrane fraction (Table 2). A higher percentage of PAP was recovered in the mitochondrial-outer-membrane fraction which had previously been washed with 0.5 M-NaCl, compared with the mitochondrial-outer-membrane fraction prepared in sucrose. The translocation of PAP to mitochondrial-outer-membrane fractions was compared with that occurring to microsomes. Translocation of PAP to microsomes was stimulated by oleate. The percentage of PAP recovered in the microsomal-membrane fraction after the translocation incubation was lower than observed for the mitochondrial-outer-membrane fraction. Washing of microsomal membranes in 0.5 M-NaCl before the translocation incubation did not stimulate PAP binding to these membranes. Very little PAP translocated to mitochondrial-outer-membrane fraction, to microsomes and to mitochondrial-inner-membrane fraction in the absence of oleate. The latter stimulated only a small translocation of PAP to the mitochondrial-inner-membrane fraction (results not shown).

The activity of the microsomal enzyme NADPH-cytochrome *c* reductase in the outer-membrane fraction (sucrose-washed) was 23.56 ± 8.89 nmol/min per mg of protein, in the outer-membrane fraction (NaCl-washed) 23.12 ± 7.57 nmol/min per mg of protein and in the microsomal fraction 165.4 ± 54.1 nmol/min per mg of protein (all means $\pm$ S.D., $n = 4$). The microsomal contamination of the outer-membrane fraction was too low to account for the translocation of PAP observed to the outer-membrane fraction.

The purity of the outer-membrane fraction with respect to the mitochondrial outer membrane could not be assessed from the outer-membrane marker monoamine oxidase, as it was subsequently found that the sonication used during the preparation of the fraction caused loss of activity of monoamine oxidase, measured with either [¹⁴C]-tyramine or -benzylamine as substrate, and solubilization of the enzyme. After correction for microsomal contamination of the outer-membrane fraction, as measured by NADPH-cytochrome *c* reductase activity, and contamination by inner membrane, as measured by succinate dehydrogenase, the specific activity of rotenone-insensitive NADH-cytochrome *c* reductase was approx. 5-fold that of whole mitochondria, showing that the outer-membrane fraction did indeed

Table 1. Effect of oleate and palmitate on the relative distribution of PAP between mitochondrial and supernatant fractions after incubation of particle-free supernatant with mitochondria

Freshly prepared mitochondrial fraction and particle-free supernatant were incubated together for 10 min at 37 °C after the additions shown. Mitochondrial and supernatant fractions were prepared after this incubation as described in the Experimental section. The results are expressed as means $\pm$ S.D. for six independent experiments. Initial protein added to the incubation was 3.02 ± 0.05 mg for mitochondrial fraction and 1.55 ± 0.17 mg for particle-free supernatant. The significance of difference between groups was calculated by using a paired *t*-test, and it is shown by **P* < 0.01 and †*P* < 0.001. PAP activity, in particle-free supernatant, in the absence of additions was 5.0 ± 2.1 nmol of diacylglycerol/min per ml (mean $\pm$ S.D., four experiments only; total PAP activity relative to control is recorded for the same four experiments). PAP activity in mitochondrial fractions, in the absence of particle-free supernatant, was 3.6 ± 2.4 % of particle-free-supernatant activity (mean $\pm$ S.D., four experiments).

Particle-free supernatant	Addition to mitochondria	Relative distribution of PAP activity (%)		Total PAP activity relative to control
		Mitochondrial fraction	Supernatant	
I	None	7 ± 3	93 ± 3	1.0
II	0.37 mM-Oleate	28 ± 10 I versus II†	72 ± 10 I versus II†	1.32 ± 0.27
III	0.75 mM-Oleate	40 ± 12 I versus III† II versus III*	60 ± 12 I versus III† II versus III*	1.32 ± 0.30
IV	0.37 mM-Palmitate	13 ± 6	87 ± 6	1.27 ± 0.24
V	0.75 mM-Palmitate	22 ± 8 I versus V* IV versus V†	78 ± 8 I versus V* IV versus V†	1.36 ± 0.22

contain mitochondrial outer membrane. Unfortunately, rotenone-insensitive NADH-cytochrome *c* reductase was also partially solubilized or insufficiently sedimented after the sonication treatment. However, the method employed of swelling and shrinking of mitochondria and subsequent sonication, and gradient centrifugation has been reported to give good separation of mitochondrial outer and inner membrane [15,21].

Translocation of PAP to phosphatidate-loaded mitochondria and microsomes

In the experiments shown in Table 3, the determination of monoamine oxidase and NADPH-cytochrome *c* reductase activities indicated that most of the mitochondria were recovered after the translocation incubation in the low-speed pellet (89.8 ± 3.4 %) and that most of the microsomes were recovered in the high-speed pellet (87.0 ± 3.6 %) (both mean $\pm$ S.D., *n* = 12).

In the present work fluoride was included in the translocation incubation to prevent the PAP from utilizing the endogenous substrate [11]. To enable measurement of the PAP bound to membranes after the translocation incubation, pellets were resuspended in sucrose which did not contain fluoride. PAP activity was measured with an exogenous ³H-labelled phosphatidate substrate and radioactivity determined with a dual-label program. Very little ¹⁴C-labelled diacylglycerol was formed, indicating that PAP preferred the artificial exogenous substrate under the assay conditions employed.

Loading the membranes with phosphatidate stimulated the translocation of PAP to membranes (Table 3). A significant increase in membrane-bound PAP occurred in the low-speed pellet when membranes, either mitochondrial or microsomal, were loaded with

phosphatidate and incubated with particle-free supernatant. The increase in PAP bound in the high-speed pellet was less pronounced.

DISCUSSION

Oleate stimulates the translocation of PAP from particle-free supernatant to mitochondria (Table 1) and to a partially purified mitochondrial-outer-membrane fraction (Table 2). Oleate was more effective than palmitate in stimulating the translocation to mitochondria (Table 1), as has been described for the translocation of PAP to microsomal membrane [5].

It was of interest to observe that there was an increased translocation of PAP to salt-washed mitochondrial-outer-membrane fraction. High salt concentrations (125 mM-KCl in 10 mM-Tris/HCl/1 mM-EDTA) remove non-specific lipid transfer protein from mitochondria [22]. Perhaps the conditions employed in the present work removed peripheral protein(s), allowing better access of PAP to the membranes. Alternatively, the salt wash may have facilitated an increased interaction of fatty acid with the membrane, which in turn enhanced the binding of PAP. Moller & Hough [6] showed that the presence of salt (54 mM-KCl) facilitated the binding of PAP to microsomes, mitochondria and plasma-membrane fractions of adipocytes. In the present work, washing of microsomes with 0.5 M-NaCl did not enhance binding of PAP. The salt was not included in the translocation incubation, however.

The results of Table 2 show that oleate is able to stimulate the translocation of PAP to the mitochondrial outer membrane *in vitro*. Although liver cells may be exposed to fatty acid concentrations of 1.5 mM [23], the concentration of unesterified fatty acid in the cell, either

Table 2. Effect of oleate on the relative distribution of PAP between membrane fractions and supernatant after incubation of particle-free supernatant with outer-mitochondrial-membrane fractions or microsomal fractions

Freshly prepared, partially purified mitochondrial outer membrane or microsomes washed in either 0.5 M-NaCl or 0.25 M-sucrose/20 mM-Hepes, pH 7.4, before resuspension in the latter medium, were incubated with particle-free supernatant for 10 min at 37 °C, in the absence or presence of 0.37 mM-oleate. After incubation the membrane and supernatant fractions were prepared as described in the Experimental section. PAP activity in particle-free supernatant in the absence of additions was 2.07 ± 0.15 , 3.40 ± 0.13 , 4.52 ± 0.12 and 2.74 ± 0.01 nmol of diacylglycerol/min per ml in the four experiments. Little or no PAP activity was detectable in the outer-membrane fractions or microsomal membranes in the absence of particle-free supernatant. In the Table the results are expressed as means $\pm$ S.D. for four experiments, except for protein concentrations for microsomal fractions. These are expressed as means $\pm$ S.D. of the first two experiments and the fourth experiment. In the third experiment microsomal protein concentration was 3.6-fold higher. The significance of difference between groups was calculated by using a paired *t* test, and it is shown by **P* < 0.05, ***P* < 0.02, ****P* < 0.01, †*P* < 0.001.

	Additions to particle-free supernatant	Relative distribution of PAP activity (%)		Specific activity of PAP in membrane fraction (nmol of diacylglycerol/min per mg of protein)	Total PAP activity relative to particle-free supernatant	Protein concn. of membrane fraction after translocation (mg/ml)
		Membrane fraction	Supernatant			
I	Mitochondrial outer membrane, sucrose-washed	7.4 ± 3.2	92.6 ± 3.2	0.276 ± 0.109	0.83 ± 0.09	5.17 ± 1.51
II	Mitochondrial outer membrane, sucrose-washed + 0.37 mM-oleate	67.6 ± 6.7 I versus II†	32.4 ± 6.7	4.380 ± 1.522 I versus II**	1.38 ± 0.35	5.17 ± 1.48
III	Mitochondrial outer membrane, NaCl-washed	11.1 ± 6.9	88.9 ± 6.9	0.362 ± 0.173	0.74 ± 0.06	4.97 ± 1.25
IV	Mitochondrial outer membrane, NaCl-washed + 0.37 mM-oleate	75.1 ± 5.4 III versus IV† II versus IV***	24.9 ± 5.4	5.032 ± 1.573 III versus IV*** II versus IV***	1.44 ± 0.28 III versus IV**	5.17 ± 1.25
V	Microsomes, sucrose-washed	7.9 ± 5.8	92.1 ± 5.8	0.318 ± 0.223	1.01 ± 0.06	4.55 ± 1.78
IV	Microsomes, sucrose-washed + 0.37 mM-oleate	49.5 ± 10.8 V versus VI*** II versus VI*	50.5 ± 10.8	2.005 ± 0.854 V versus VI*	1.12 ± 0.23	4.98 ± 1.48
VII	Microsomes, NaCl-washed	5.8 ± 2.7	94.2 ± 2.7	0.208 ± 0.111	0.90 ± 0.08	4.65 ± 1.48
VIII	Microsomes, NaCl-washed + 0.37 M-oleate	50.8 ± 10.0 VII versus VIII*** IV versus VIII*	49.2 ± 10.0	1.977 ± 0.758 VII versus VIII** IV versus VIII*	1.12 ± 0.08 VII versus VIII*	4.89 ± 1.48

Table 3. PAP activity in membrane fractions after incubation of particle-free supernatant with mitochondria, microsomes, mitochondria loaded with phosphatidate and microsomes loaded with phosphatidate

Particle-free supernatant was incubated with various additions for 10 min at 37 °C. Low-speed and high-speed pellets were then prepared as described in the Experimental section. Results are shown as means $\pm$ range of duplicate determinations, and are expressed as nmol of diacylglycerol/min per fraction. PAP activity in particle-free supernatant in the absence of F⁻ was 2.00 ± 0.35 nmol/min per ml (Expt. 1). This activity was not measured in Expt. 2. Mitochondrial phosphatidate concentration (Expt. 2) was 26.8 ± 6.1 nmol/mg of protein. Microsomal phosphatidate concentration (Expt. 2) was 70.0 ± 2.8 nmol/mg of protein.

Additions to particle-free supernatant	Expt.	PAP activity	
		Low-speed pellet	High-speed pellet
Mitochondria	1	0.226 ± 0.028	0.669 ± 0.080
	2	0.181 ± 0.006	1.102 ± 0.056
Mitochondria, phosphatidate-loaded	1	1.829 ± 0.003	0.868 ± 0.002
	2	0.929 ± 0.060	0.888 ± 0.036
Microsomes	1	0.176 ± 0.001	0.349 ± 0.035
	2	0.192 ± 0.001	0.580 ± 0.023
Microsomes, phosphatidate-loaded	1	0.999 ± 0.019	0.655 ± 0.007
	2	0.597 ± 0.038	0.649 ± 0.006

in the cytoplasm or in association with membranes, is not known. Spermine enhances the effect of oleate on the translocation of PAP to microsomal membranes [24], so that in the cell the presence of polyamines may facilitate translocation of PAP at lower fatty acid concentrations than are required *in vitro*. Fatty acid is metabolized *in vivo* to acyl-CoA, which is more potent than unesterified fatty acid in promoting translocation to microsomes *in vitro* [5]. The acyl-CoA is subsequently metabolized in the cell to phosphatidate. The latter could act as the signal to promote PAP binding to membranes *in vivo*. The translocation of PAP *in vitro* to microsomal membranes containing phosphatidate has been demonstrated [25]. In the present work it was shown that PAP also translocates to mitochondria containing phosphatidate (Table 3).

More PAP was observed to bind to mitochondria loaded with phosphatidate than to microsomes loaded with phosphatidate, even though the concentration of phosphatidate in the microsomes exceeded that in mitochondria (Table 3). If the mitochondrial phosphatidate was located in the outer membrane, the effective concentration of phosphatidate in that membrane would be higher than that of phosphatidate in microsomal membranes, as the mitochondrial outer membrane comprises only a small proportion of the total protein of whole mitochondria. If PAP binding is dependent on phosphatidate concentration, then location of mitochondrial phosphatidate in the outer membrane would explain the results observed (Table 3).

The high recovery of PAP activity in the high-speed pellet after incubation of unlabelled mitochondria with particle-free supernatant was surprising (Table 3). This suggests that some fatty acid was present in the particle-free supernatant which promoted translocation of PAP to mitochondrial-outer-membrane fragments in this fraction, or to contaminating fragments of endoplasmic reticulum. The former suggestion may apply, as less PAP was recovered in the high-speed pellet when unlabelled microsomes were incubated with particle-free supernatant (Table 3). Alternatively, polyamines [24] or some unknown factor in the particle-free supernatant may have promoted the translocation of PAP to the membrane fragments recovered in the high-speed pellet after incubation of particle-free supernatant with unlabelled mitochondria.

It was interesting to observe that the PAP bound in the low-speed pellet increased, relative to control, when particle-free supernatant was incubated with microsomes loaded with phosphatidate. This result could be explained by some transfer of phosphatidate from microsomes to mitochondria. This has been observed to occur in both the absence [26] and the presence of cytoplasm [27]; in the latter case a transfer protein may facilitate the movement of phosphatidate to mitochondria.

When hepatocytes were incubated with fatty acid, the total activity of PAP increased and an increased proportion of the enzyme was found to be membrane-bound [1]. In the present work there was an increase in the total activity of PAP, derived from particle-free supernatant, in the presence of mitochondria and fatty acids (Table 1) or in the presence of mitochondrial-outer-membrane fraction and oleate (Table 2). Experiments designed to measure the translocation of PAP to mitochondria of hepatocytes in culture have not so far been successful. To obtain a good separation of mitochondria and endo-

plasmic reticulum, with complete lysis of cells, required sonication and homogenization of cell suspensions (results not shown). It was found that sonication of mitochondria to which PAP had been translocated *in vitro* caused loss of PAP activity. It is also possible that the homogenization process generated enough shear force and/or heat to dissociate the enzyme from the outer membrane of mitochondria.

Although translocation of PAP to mitochondria has not been demonstrated *in vivo*, the results of the experiments *in vitro* described above suggest that it could occur. PAP does translocate to rat hepatocyte cell membranes *in vivo* [1-4] and to cell membranes of a cell line from human lung [28]. The translocation of CTP:phosphocholine cytidyltransferase from cytosol to membranes in rat hepatocytes [29] and in HeLa cells [30] is stimulated by oleate in a similar manner to the translocation of PAP to microsomes [5] and mitochondria (Table 1). Extracellular stimulation can modify the translocation *in vivo* of PAP [1-4], CTP:phosphocholine cytidyltransferase [29-31], protein kinase C [32,33] and phospholipase A₂ [33]. For each of these enzymes the activity is enhanced when the enzyme is membrane-bound. The activity of hexokinase is enhanced when this enzyme is bound to mitochondria [34]. In contrast with PAP and CTP:phosphocholine cytidyltransferase, hexokinase is detached from mitochondria by fatty acid.

At present there is a paradox: mitochondria have an apparently active glycerophosphate acyltransferase, responsive to hormones [35], yet they display an apparent inability to synthesize phosphoglycerides, with the exception of cardiolipin at a low rate [36]. Translocation of PAP to mitochondria offers a possible solution. Mitochondrial phosphatidate may be readily converted by PAP into diacylglycerol, and this lipid could be rapidly transferred to other membranes. It is not known if this occurs in hepatocytes. In fibroblasts, when a fluorescent analogue of phosphatidate was inserted into the plasma membrane, fluorescence was found in internal membrane sites only after the conversion into the diacylglycerol derivative [37].

Indirect evidence supports an important role for mitochondrial phosphatidate in cell glycerolipid biosynthesis. The mitochondrial system of phosphatidate biosynthesis may contribute to the positional specificity of glycerolipids in the intact cell [10,38]. Most cell lipids have a saturated fatty acid residue at position 1 and an unsaturated fatty acid residue at position 2 on the glycerol backbone [39,40]. The mitochondrial glycerophosphate acyltransferase has a high selectivity for saturated acyl-CoA, particularly palmitoyl-CoA [39,41]. An inverse relationship was found between the acylating capacity of mitochondria and the amount of unsaturated fatty acid at position 1 in choline phosphoglycerides in several types of cultured cells [42]. The mitochondrial glycerophosphate acyltransferase is low or absent in ascites-tumour cells, which have unusual positioning of saturated fatty acids in certain phosphoglycerides [43]. These observations [42,43] could be explained if diacylglycerol, formed from mitochondrial phosphatidate, was subsequently utilized by microsomal enzymes for phosphoglyceride biosynthesis.

The primary site of incorporation of [³H]palmitate into liver lipid was shown by electron microscopy to be the endoplasmic reticulum; subsequently mitochondria became radiolabelled. [³H]Glycerol was incorporated

into lipid in both the endoplasmic reticulum and mitochondria 5 min after injection [44]. It was calculated that the mitochondrial fraction contributed about 34% of liver homogenate biosynthesis when a palmitoyl-CoA-generating system and *sn*-glycerol 3-phosphate were employed in the assay [7]. Since neither acyl-CoA synthetase [45,46] nor *sn*-glycerol 3-phosphate concentration [46] is thought to limit the esterification of fatty acid *in vivo*, mitochondria may indeed have a significant role in liver lipid biosynthesis; the extent of this contribution *in vivo* is not yet known, however.

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