Biochem. J. (1979) **177**, 283–288
Printed in Great Britain

Evidence for the Existence of Isoenzymes of Glycerol Phosphate Acyltransferase

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(*Received* 11 *August* 1978)

Subcellular-fractionation studies confirmed previous findings that rat liver glycerol phosphate acyltransferase was located in both mitochondria and the microsomal fraction. Studies of the two activities revealed several differences between them. The mitochondrial enzyme had a lower K_m for *sn*-glycerol 3-phosphate and was more resistant to heat inactivation than was the microsomal enzyme. Some preparations of the mitochondrial enzyme were inhibited by high concentrations of glycerol phosphate. The mitochondrial enzyme was not inactivated by thiol-group reagents, whereas the microsomal enzyme was very rapidly inactivated by these compounds. However, the microsomal enzyme could be specifically protected against this inactivation by low concentrations of palmitoyl-CoA. The results indicate the existence of distinct isoenzymes of glycerol phosphate acyltransferase with different intracellular locations.

The biosynthesis of phosphatidate by the acylation of *sn*-glycerol 3-phosphate was first reported by Kornberg & Pricer (1953). Since then it has been shown that dihydroxyacetone phosphate can also give rise to phosphatidate by acylation, reduction of the 1-acyldihydroxyacetone phosphate and a further acylation (Hajra, 1968; La Belle & Hajra, 1972). There has been some controversy as to the relative importance of the two pathways in the synthesis of glycerolipids [for a recent review, see van Golde & van den Bergh (1977)]. It is, however, clear that the esterification pathways divert fatty acids from oxidation to storage and thus occupy a key position in the metabolism of fatty acids. The properties of the acyltransferases involved in these pathways may therefore be of considerable importance in the regulation of lipid metabolism.

Little is known about the properties of the enzymes involved in the esterification processes. The biosynthesis of phosphatidate from *sn*-glycerol 3-phosphate involves two distinct enzymes, glycerol phosphate acyltransferase (acyl-CoA–*sn*-glycerol 3-phosphate *O*-acyltransferase, EC 2.3.1.15) and 1-acylglycerol phosphate acyltransferase (Yamashita & Numa, 1972). Some early disputes as to the subcellular location of glycerol phosphate acyltransferase in liver have now been resolved, and it is generally agreed that the enzyme is located in both the mitochondria and the microsomal fraction (Stoffel & Schiefer, 1968; Shepard & Hubscher, 1969; Daae & Bremer, 1970; Monroy *et al.*, 1972; Davidson & Stanacev, 1974; Jones & Hajra, 1977). The mitochondrial activity is located in the outer membranes (Stoffel & Schiefer, 1968; Zborowski & Wojtczak, 1969; Daae & Bremer, 1970; Monroy *et al.*, 1972).

Several observations have been made that suggest that there may be differences between the mitochondrial and microsomal glycerol phosphate acyltransferase activities. Firstly, the products of the two acylation systems may be different. Under conditions in which the isolated microsomal fraction produces mainly phosphatidate, mitochondria produce largely lysophosphatidate (Daae & Bremer, 1970; Daae, 1972, 1973). However, mitochondria can produce largely phosphatidate in some circumstances (Zborowski & Wojtczak, 1969; Monroy *et al.*, 1972), and it seems that the incubation conditions can play a large part in determining the final reaction product (Monroy *et al.*, 1972; Bjerve *et al.*, 1976).

Secondly, the two acylation systems differ in their responses to thiol-group reagents. The microsomal glycerol phosphate acyltransferase from rat liver is almost completely inhibited by reagents such as iodoacetamide and *N*-ethylmaleimide, whereas the mitochondrial activity is slightly stimulated by these reagents (Lands & Hart, 1965; Monroy *et al.*, 1972; Bates *et al.*, 1977). Thirdly, the mitochondrial glycerol phosphate acyltransferase is affected much more by insulin or diabetes than is the microsomal activity (Bates & Saggerson, 1977; Bates *et al.*, 1977).

There is thus some indirect evidence that, in liver, the mitochondrial and microsomal glycerol phosphate acyltransferases may be distinct. Although solubilization of the two activities has been reported

(Yamashita & Numa, 1972; Monroy *et al.*, 1973), the proteins have not been purified, and there is thus no direct evidence that shows them to be chemically distinct. The present results indicate that the mitochondrial and microsomal acyltransferases are distinct isoenzymes.

Experimental

Materials

Glycerokinase, *sn*-glycerol 3-phosphate, oxaloacetic acid, acetyl-CoA and glucose 6-phosphate were obtained from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Bovine serum albumin, palmitoyl-DL-carnitine hydrochloride, *rac*-glycerol 3-phosphate, 5,5′-dithiobis-(2-nitrobenzoic acid), methyl palmitate and glycerol phosphate dehydrogenase were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Iodoacetamide, *N*-ethylmaleimide, 2-mercaptoethanol and dithiothreitol were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. [1(3)-^{3}H]Glycerol was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

sn-[^{3}H]Glycerol 3-phosphate was prepared from [^{3}H]glycerol by the method of Smith & Hubscher (1966). Carnitine palmitoyltransferase (palmitoyl-CoA–L-carnitine *O*-palmitoyltransferase, EC 2.3.1.21) was prepared from ox liver by the method of Edwards (1973) up to and including the alumina-gel step. The method involves precipitation at pH 5.5, $(NH_4)_2SO_4$ fractionation, chromatography on DEAE-cellulose and fractionation on alumina gel. The purified material had a specific activity of 2.0 units/mg and contained no detectable palmitoyl-CoA hydrolase activity. It was stored in 20mM-potassium phosphate, pH 7.5, containing 40% (v/v) glycerol at −20°C, and was stable for several months under these conditions.

Assay procedures

Glycerol phosphate acyltransferase was assayed by a modification of the method of Daae & Bremer (1970). The incubation comprised, in a total volume of 0.12ml, 50mM-Tris/HCl, pH 7.5, 100μM-CoASH, 5mM-$MgCl_2$, 6mg of bovine serum albumin (fatty acid-free)/ml, 0.5mM-*sn*-[^{3}H]glycerol 3-phosphate (specific radioactivity 2×10^6 c.p.m./μmol), 0.4mM-palmitoyl-DL-carnitine, 0.05 unit of carnitine palmitoyltransferase and 15mM-mercaptoethanol. This mixture was preincubated at 30°C for 5min and the reaction was started by the addition of 0.02–0.5mg of the sample being assayed. After 10min at 30°C, the incubation was stopped by the addition of 0.4ml of water-saturated butanol. After centrifugation the butanol layer was washed with 2ml of butanol-saturated water containing 1mM-*rac*-glycerol 3-phosphate. Finally a 0.1ml portion of the butanol layer was removed for determination of radioactivity. Control experiments showed that the incorporation of glycerol phosphate into butanol-soluble material was linear with time up to 15min, and with enzyme concentration in the assay up to 0.3/unit/ml.

Carnitine palmitoyltransferase, citrate synthase and glucose 6-phosphatase were assayed by the methods of West *et al.* (1971), Srere (1969) and Swanson (1955) respectively. Protein was determined by the method of Bradford (1976).

Preparation of subcellular fractions

Male Wistar rats (approx. 200g) deprived of food overnight were used in all experiments. Subcellular fractions of liver were prepared by the method of de Duve *et al.* (1955). The tissue was homogenized, and the fractions were resuspended in 0.25M-sucrose/10mM-Tris/HCl (pH 7.4)/1mM-EDTA/15mM-mercaptoethanol. For experiments in which the reaction of fractions with thiol-group reagents was studied, the mercaptoethanol was replaced by 1mM-dithiothreitol. The procedure gave rise to the fractions N (nuclei), M (heavy mitochondria), L (light mitochondria, lysosomes, peroxisomes), P (microsomal) and and S (supernatant). The terms mitochondria and microsomal fraction used hereafter refer to fractions M and P respectively.

Reaction with thiol-group reagents

For these experiments, subcellular fractions were resuspended in buffer containing 1mM-dithiothreitol at a protein concentration of 2–4mg/ml. The thiol-group reagent was added to give a concentration of 10mM, and the mixtures were incubated at 0°C. Samples (20μl) were removed at various times and assayed in the presence of excess mercaptoethanol (15mM). Where protection studies were carried out, the protecting agent was added just before the thiol-group reagent. Control experiments were carried out to show that the protecting agents were not destroyed during the incubations.

Results and Discussion

Fractionation studies

In agreement with previous workers (see the introduction), it was found that glycerol phosphate acyltransferase activity was located in both the mitochondria and the microsomal fraction. In a series of eight experiments the recovery of glycerol phosphate acyltransferase activity was in the range 90–110%. The specific activity of the mitochondrial enzyme was 1.8 ± 0.4nmol/min per mg and that of the microsomal enzyme was 1.7 ± 0.5nmol/min per mg. Studies with the marker enzymes citrate synthase and glucose 6-phosphatase showed that the contamination of mitochondria by the microsomal fraction was less than 10%, and that of the microsomal fraction by

mitochondria was less than 8%. The properties of these two fractions were examined further.

Kinetic studies

The optimal assay conditions for the enzyme in the mitochondrial and microsomal fractions were very similar. In each case the presence of bivalent cations (1–5mM) stimulated the activity by up to 50%: Mg^{2+}, Mn^{2+} and Ca^{2+} were equally effective and 5mM-$MgCl_2$ was routinely included in the assay. The enzyme in each fraction had a broad pH optimum in the range pH7.5–8.0. The optimal concentrations of serum albumin (6mg/ml) and palmitoylcarnitine (0.4mM) were very similar for each fraction. In each case the reaction products, which were identified as described by Daae & Bremer (1970), were mainly (>80%) phosphatidate, together with small amounts of lysophosphatidate and diacylglycerol.

The mitochondrial and microsomal glycerol phosphate acyltransferase activities both responded hyperbolically to *sn*-glycerol 3-phosphate, as shown in Fig. 1. The K_m of the microsomal enzyme for this substrate (0.33±0.06mM, $n = 5$) was significantly ($P < 0.001$, Student's t test) higher than that of the mitochondrial enzyme (0.18±0.05mM, $n = 5$). With some preparations of the mitochondrial activity, it was found that concentrations of *sn*-glycerol 3-phosphate above 0.7mM gave high substrate inhibition. At 2.0mM-glycerol phosphate, up to 70% inhibition of the activity at 0.5mM-glycerol phosphate was observed. This phenomenon has been observed with five out of fourteen preparations of the mitochondrial activity so far studied, but has not yet been found with any microsomal preparation. The K_m of preparations that show high substrate inhibition for glycerol phosphate is not significantly different from that of preparations that do not show this behaviour. The reason for this odd behaviour is not known, but to avoid possible high substrate inhibition, the concentration of glycerol phosphate used in the standard assay was 0.5mM.

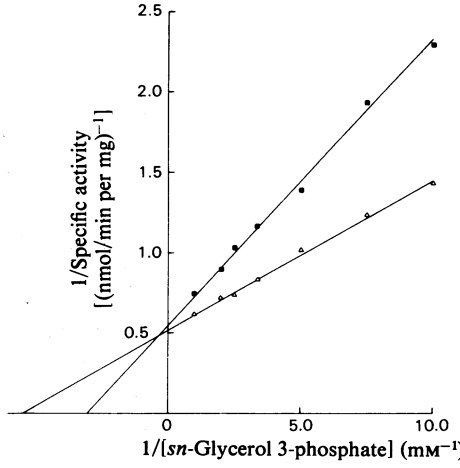

Fig. 1. *Double-reciprocal plots for the response of glycerol phosphate acyltransferase to sn-glycerol 3-phosphate*
Mitochondria (△) and microsomal fraction (■) were assayed as described in the Experimental section, except that the concentration of *sn*-glycerol 3-phosphate was varied as indicated. Rates are expressed as specific activities in nmol/min per mg of protein.

It is not easy to compare the kinetic results shown here with those obtained in previous studies of glycerol phosphate acyltransferase because, in a number of cases, very different assay conditions have been used. However, the results obtained here differ in several respects from those of other workers who have also used the generation of palmitoyl-CoA from palmitoylcarnitine in the assay of the enzyme. Firstly, Daae & Bremer (1970) reported that the K_m of the mitochondrial acyltransferase for glycerol phosphate was 2mM, some 10-fold higher than the value found here. This difference may have resulted from the fact that Daae & Bremer (1970) used racemic glycerol phosphate, whereas the pure isomer, *sn*-glycerol 3-phosphate, was used in the present study. Secondly, previous reports showed that the mitochondrial acylation system produced more lysophosphatidate than phosphatidate (Daae & Bremer, 1970; Daae, 1972, 1973), whereas quite the reverse was found here. It is known that the nature of the product is dependent on the concentration of serum albumin in the assay (Monroy *et al.*, 1972), but in this respect the conditions used here were similar to those used in the previous studies (Daae & Bremer, 1970; Daae, 1972, 1973). It is more likely that the difference results from the concentration of glycerol phosphate used in the assay; it has previously been noted that assays of the mitochondrial system using low concentrations of glycerol phosphate give rise to phosphatidate, whereas assays at high concentrations give lysophosphatidate (Monroy *et al.*, 1972; Bjerve *et al.*, 1976). The present results are in agreement with those of Zborowski & Wojtczak (1969), who also used 0.5mM-glycerol phosphate and who found that the main product of the mitochondrial system was phosphatidate. It is interesting to speculate that the production of lysophosphatidate rather than phosphatidate by the mitochondrial system at high glycerol phosphate concentrations may be related to the high substrate inhibition that was sometimes observed in the present study, although the cause of this inhibition is not known at present.

Heat-inactivation studies

The susceptibility of liver glycerol phosphate acyltransferase activity to thermal inactivation at

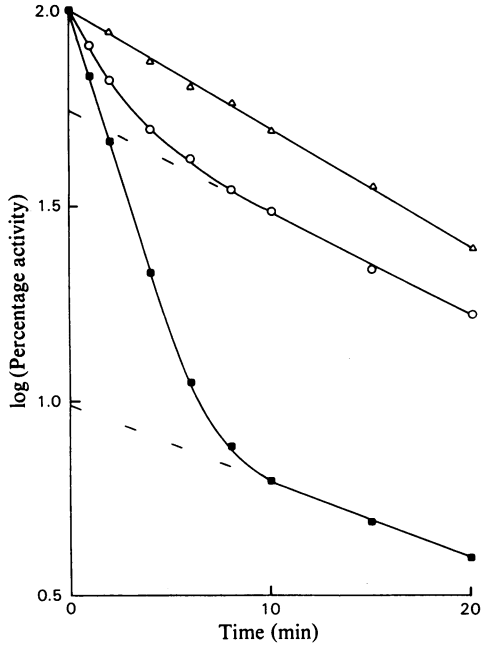

Fig. 2. *Heat-inactivation of glycerol phosphate acyltransferase*

Samples (0.4 ml) of each fraction were rapidly brought to 51°C. At various times, 0.05 ml portions were removed, cooled and assayed for glycerol phosphate acyltransferase as described in the Experimental section. The results are expressed as plots of log(percentage activity remaining) against time for the mitochondria (△), microsomal fraction (■) and the original homogenate (○).

51°C was examined. Typical semilogarithmic plots for the inactivation of the enzyme in a crude liver extract and in the mitochondrial and microsomal fractions are shown in Fig. 2. The mitochondrial fraction appears to comprise a single component relatively resistant to heat-inactivation. The microsomal fraction on the other hand comprises about 90% of a heat-labile component with about 10% of a less labile component. The results for the crude extract suggest that it contains about 55% of a relatively heat-stable component and about 45% of a heat-labile component. In a further experiment, equal volumes of the mitochondrial and microsomal fractions containing equal activities were mixed and incubated at 51°C. The mixture behaved as a simple combination of approximately equal amounts of two components with markedly different heat-stabilities, showing that neither fraction affected the stability of the other (results not shown). Thus there seems to be some inherent difference between the glycerol phosphate acyltransferases of mitochondrial and microsomal fractions that is reflected in a difference between their heat-stabilities. This could be either a difference in the enzyme protein(s) or a difference in the environment of the protein(s).

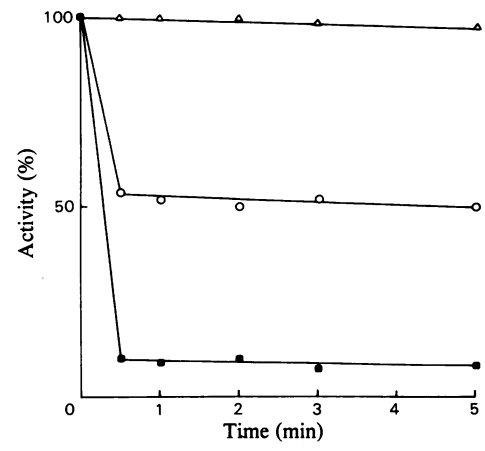

Fig. 3. *Sensitivity of glycerol phosphate acyltransferase to inhibition by N-ethylmaleimide*

Reactions with *N*-ethylmaleimide were carried out as described in the Experimental section. Activities are expressed as percentages of that of the uninhibited enzyme for the mitochondria (△), microsomal fraction (■) and the original homogenate (○).

Sensitivity to thiol-group reagents

Typical time courses for the inactivation of glycerol phosphate acyltransferase in a crude liver extract and in the mitochondrial and microsomal fractions by 10mM-*N*-ethylmaleimide are shown in Fig. 3. In agreement with previous workers (Monroy *et al.*, 1972; Bates *et al.*, 1977), the results show a very distinct difference between the mitochondrial enzyme, which is inactivated only very slowly, and the microsomal enzyme, which is inactivated by 90% within the first 30s of the reaction. The crude extract seems to contain approximately equal amounts of two components; one component is unaffected by the reagent, whereas the other is very rapidly inactivated.

The sensitivity of the microsomal glycerol phosphate acyltransferase to thiol-group reagents could result from their reaction with either the enzyme protein(s) or some other membrane component. It does not necessarily imply that the microsomal glycerol phosphate acyltransferase is chemically distinct from the mitochondrial enzyme. In further experiments, the effects of reagents that might protect the microsomal enzyme against inactivation were examined. In these experiments *N*-ethylmaleimide was replaced by iodoacetamide, which gave a slower, measurable rate of inactivation. Time courses for the inactivation of the enzyme by

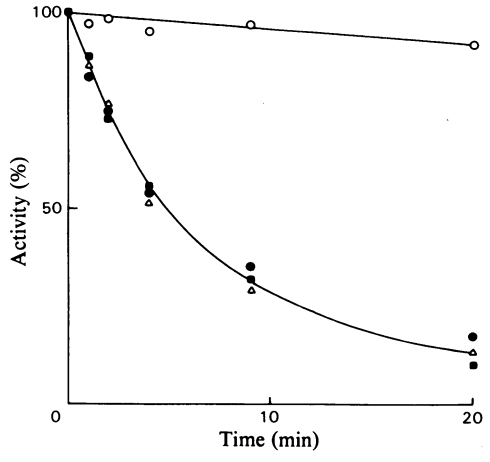

Fig. 4. *Protection of microsomal glycerol phosphate acyltransferase against inactivation by iodoacetamide*
The microsomal fraction was incubated with iodoacetamide as described in the Experimental section. The following reagents were also present: none (△), 0.2mM-acetyl-CoA (●); 50μM-methyl palmitate (■); 50μM-palmitoyl-CoA (○).

Table 1. *Comparison of the sensitivities of glycerol phosphate acyltransferase to heat treatment and to N-ethylmaleimide*
Subcellular fractions were subjected to heat treatment as described in Fig. 2, and to reaction with *N*-ethylmaleimide as described in Fig. 3. The heat-treatment studies were interpreted in terms of the presence of two components of different stabilities and the estimates of the proportions of these were made from semilogarithmic plots (cf. Fig. 2). Estimates of the proportion of enzyme activity sensitive to *N*-ethylmaleimide were made from time-course experiments (cf. Fig. 3). Results are expressed as means ±S.D. (*n*). In no case was the proportion of activity that was heat-labile significantly different from the proportion sensitive to *N*-ethylmaleimide ($P<0.05$). N, M, L and P are defined in the text.

Fraction	Heat-labile activity (%)	Activity sensitive to *N*-ethylmaleimide (%)
Homogenate	45± 8 (4)	50±5 (7)
N	24± 6 (3)	19±5 (3)
M	3± 4 (4)	4±3 (6)
L	45±10 (3)	41±5 (5)
P	90± 7 (4)	92±5 (7)

10mM-iodoacetamide under various different conditions are shown in Fig. 4. The rate of inactivation was not affected by 5mM-*sn*-glycerol 3-phosphate (results not shown), 0.2mM-acetyl-CoA or 50μM-methyl palmitate, but it was markedly decreased by 50μM-palmitoyl-CoA.

Crude liver extracts thus contain two distinct glycerol phosphate acyltransferase activities, which can be distinguished by their heat-stabilities and sensitivities to thiol-group reagents. In several subcellular-fractionation experiments the relative proportions of the two forms in the various fractions were assessed by heat-inactivation studies (cf. Fig. 2) and reaction with *N*-ethylmaleimide (cf. Fig. 3). The results are shown in Table 1. There is good agreement between the estimates of the proportions of the two components obtained by the two different methods. This supports the idea that the glycerol phosphate acyltransferase that is relatively heat-labile is also sensitive to *N*-ethylmaleimide, whereas the more heat-stable activity is resistant to thiol-group reagents.

The observation that palmitoyl-CoA protects the microsomal glycerol phosphate acyltransferase against inactivation by thiol-group reagents (the present study; Husbands & Lands, 1970) is very significant, because it implies that the enzyme contains a reactive thiol group at or near its active centre. This suggests that the catalytic mechanism may involve an acyl-enzyme intermediate and it may afford a means of labelling the enzyme specifically. The possibility that the mitochondrial acyltransferase also contains a potentially reactive thiol group that is masked by endogenous palmitoyl-CoA seems very unlikely, because the mitochondria contain significant palmitoyl-CoA hydrolase activity (H. G. Nimmo, unpublished work). Taken with the heat-inactivation studies, the present results thus constitute convincing evidence that the mitochondrial and microsomal glycerol phosphate acyltransferases are chemically distinct.

Glycerolipid synthesis can also occur via the acylation of dihydroxyacetone phosphate (see the introduction). It has recently been shown that a dihydroxyacetone phosphate acyltransferase is located in the peroxisomes, distinct from either of the glycerol phosphate acyltransferases (Jones & Hajra, 1977). This does not, of course, imply that the enzymes studied here cannot use dihydroxyacetone phosphate as a substrate. Indeed, Schlossman & Bell (1977) have shown that liver microsomal glycerol phosphate acyltransferase can acylate dihydroxyacetone phosphate, albeit poorly. It remains to be seen whether this is also true for the mitochondrial glycerol phosphate acyltransferase.

The relative contributions of the glycerol phosphate and dihydroxyacetone phosphate pathways of glycerolipid synthesis in liver is not fully understood, although it is known that dihydroxyacetone phosphate is an obligatory precursor of the glycerol moiety of ether lipids (van Golde & van den Bergh, 1977). The peroxisomal dihydroxyacetone phosphate acyltransferase (Jones & Hajra, 1977) may be involved in the latter process. However, the functional significance of the existence of isoenzymes of glycerol

phosphate acyltransferase is not yet clear. In view of the fact that insulin and diabetes affect the mitochondrial activity rather than the microsomal one (Bates & Saggerson, 1977; Bates *et al.*, 1977), the two enzymes may have different regulatory properties.

This work was supported by a grant from the Medical Research Council (U.K.). I thank Dan Brolly for excellent technical assistance.

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