Rat Adipose-Tissue Glycerol Phosphate Acyltransferase Can be Inactivated by Cyclic AMP-Dependent Protein Kinase

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Rat adipose-tissue glycerol phosphate acyltransferase can be inactivated in a phosphorylation reaction catalysed by cyclic AMP-dependent protein kinase and reactivated by treatment with alkaline phosphatase. These results suggest that phosphorylation of glycerol phosphate acyltransferase may be involved in the hormonal control of esterification.

The importance of white adipose tissue in mammalian metabolism lies in its ability to store fatty acids as triacylglycerols and to release them again when required. These processes are under hormonal control. The discovery that hormone-sensitive lipase can be activated by cyclic AMP-dependent protein kinase (Corbin *et al.*, 1970; Huttunen *et al.*, 1970) has led to considerable progress in our understanding of the hormonal regulation of lipolysis (for a recent review see Steinberg, 1976). Hormonal control of fatty acid synthesis may involve pyruvate dehydrogenase (Jungas, 1970; Coore *et al.*, 1971), acetyl-CoA carboxylase (Halestrap & Denton, 1973) or fatty acid synthetase (Volpe & Vagelos, 1973). In addition to these mechanisms, the esterification pathway is itself under hormonal control (Denton & Halperin, 1968; Sooranna & Saggerson, 1975). One of the enzymes that may be rate-limiting in this pathway is glycerol phosphate acyltransferase (acyl-CoA-*sn*-glycerol 3-phosphate 1-acyltransferase, EC 2.3.1.15) and it has recently been reported that the administration of adrenalin to isolated rat fat cells causes a decrease in the activity of this enzyme (Sooranna & Saggerson, 1976). However, no evidence was presented as to the mechanism of this effect. In this paper we present results that show that rat adipose-tissue glycerol phosphate acyltransferase can be inactivated by cyclic AMP-dependent protein kinase.

Experimental

Materials

ATP, NADPH, cyclic AMP, CoA, *sn*-glycerol 3-phosphate and calf intestinal alkaline phosphatase were obtained from Boehringer Corp. (London), Lewes, Sussex, U.K. Calf thymus histone (type II-A), horse heart cytochrome *c*, *rac*-glycerol 3-phosphate, palmitoyl-DL-carnitine hydrochloride and dithiothreitol were purchased from Sigma (London)

Chemical Co., Poole, Dorset, U.K. *sn*-[U-¹⁴C]-Glycerol 3-phosphate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Carnitine palmitoyltransferase (EC 2.3.1.2.1) was prepared from bovine liver by the method of Edwards (1973) up to and including the alumina-gel step. Protein phosphatase III from rabbit skeletal muscle was prepared as described by Antoniw *et al.* (1977). Cyclic AMP-dependent protein kinase from rabbit skeletal muscle (isoenzyme I) was prepared and treated to remove glycogen synthase kinase 2 as described by Nimmo *et al.* (1976). The inhibitor protein of cyclic AMP-dependent protein kinase was prepared as described by Walsh *et al.* (1971) up to and including the trichloroacetic acid-precipitation step. It was dialysed in 50mM-Tris/HCl, pH 7.5, before use.

Methods

Assay methods

The assay of glycerol phosphate acyltransferase involved the generation of palmitoyl-CoA from palmitoylcarnitine (Daae & Bremer, 1970). The incubation comprised, in a total volume of 0.12 ml, 50mM-Tris/HCl, pH 7.5, 100 μ M-CoA, 5mM-MgCl₂, 6mg of bovine serum albumin (fatty acid-free)/ml, 0.5mM-[¹⁴C]-*sn*-glycerol 3-phosphate (specific radioactivity 6×10^6 d.p.m./ μ mol), 0.4mM-palmitoyl-DL-carnitine, 0.05 units of carnitine palmitoyltransferase (specific activity 2.0 units/mg; 1 unit of activity is the amount of enzyme required to convert 1 μ mol of substrate/min) and 15mM-2-mercaptoethanol. This mixture was preincubated at 30°C for 5 min and the reaction was started by the addition of 0.01–0.02 mg of microsomal protein. After 10 min at 30°C the incubation was stopped by the addition of 0.4 ml of water-saturated butanol. After centrifugation the butanol layer was washed with 2 ml of butanol-saturated water containing 1mM-*rac*-glycerol 3-phos-

phate. Finally 0.1 ml samples of the butanol layer were removed for determination of radioactivity.

Control experiments showed that the incorporation of glycerol phosphate into butanol-soluble material was linear with time up to 15 min and with microsomal protein concentration up to 0.3 mg/ml. The concentrations of serum albumin and palmitoylcarnitine were optimal, whereas that of *sn*-glycerol 3-phosphate was slightly suboptimal, the K_m for this substrate being 0.2 mM in these conditions. Blank rates in the absence of microsomal fraction or palmitoylcarnitine were negligible. The products of the acylation system, identified as described by Daae & Bremer (1970), were mainly (>80%) phosphatidate with small amounts of lysophosphatidate and diacylglycerol. The butanol extraction procedure gave quantitative recoveries of the acylated products, as was shown by Daae & Bremer (1970).

Cyclic AMP-dependent protein kinase was assayed by using the cellulose phosphate paper method of Witt & Roskoski (1975). The inhibition of this enzyme by the inhibitor protein was also tested in this system. NADPH-cytochrome *c* reductase was assayed in the presence of 0.25 mM-KCN as described by Masters *et al.* (1967). Protein was assayed by the method of Bradford (1976). *sn*-Glycerol 3-phosphate was assayed as described by Hohorst (1963).

Preparation of microsomal fraction

Male Wistar rats (180–200 g), starved overnight, were killed by cervical dislocation and the epididymal fat pads were removed. The fat pads were rinsed in ice-cold homogenization buffer (0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 1 mM-dithiothreitol) and homogenized in 5 vol. of this buffer in a Dounce-type homogenizer. The homogenate was centrifuged at 10000 *g* for 10 min and the infranatant was removed and filtered through glass wool. It was then centrifuged at 100000 *g* for 60 min and the microsomal pellet was resuspended in homogenization buffer to give a protein concentration of 1 mg/ml.

Inactivation of glycerol phosphate acyltransferase

The standard incubation mixture contained, in a total volume of 0.2 ml, 0.1 ml of resuspended microsomal fraction, 5 mM-ATP, 10 mM-MgCl₂, 10 μ M-cyclic AMP and 0.05 mg of cyclic AMP-dependent protein kinase/ml. Where indicated the inhibitor protein (0.7 mg/ml) was also present. The mixture was incubated at 30°C and the reaction was started by the addition of ATP. Samples (20 μ l) were removed and assayed for glycerol phosphate acyltransferase. These assays gave linear time courses, which indicates that inactivation did not continue

during the assay. Inhibitor protein (0.7 mg/ml) gave over 99% inhibition of 0.05 mg of cyclic AMP-dependent protein kinase/ml assayed by using histone as a substrate.

Reactivation studies

Glycerol phosphate acyltransferase was inactivated by incubation for 30 min in the presence of ATP, MgCl₂, cyclic AMP and cyclic AMP-dependent protein kinase as described above. The reaction was stopped by the addition of EDTA to 15 mM and the mixture was dialysed at 4°C overnight against 500 ml of 50 mM-Tris/HCl, pH 8.5, containing 1 mM-EDTA and 1 mM-dithiothreitol. The dialysed enzyme preparation was incubated at 30°C with the additions indicated in the text and samples were assayed for glycerol phosphate acyltransferase. In experiments in which alkaline phosphatase was used (see below) less than 15% of the *sn*-glycerol 3-phosphate was hydrolysed by the phosphatase during the assay of glycerol phosphate acyltransferase.

Results and Discussion

When rat adipose-tissue microsomal fractions were incubated with cyclic AMP-dependent protein kinase, cyclic AMP, ATP and MgCl₂ the activity of glycerol phosphate acyltransferase decreased until, after 20 min, a limiting value of 10–15% of the original activity remained (Fig. 1). Further additions of ATP, cyclic AMP or cyclic AMP-dependent protein kinase after 30 min caused no extra decrease in activity. The average inhibition observed in a series of eight experiments was $86 \pm 9\%$ (mean $\pm$ s.d.). The activity of the microsomal enzyme NADPH-cytochrome *c* reductase was not affected by incubation in these conditions.

Some further experiments were carried out to identify the components necessary for inactivation of glycerol phosphate acyltransferase. No inactivation was observed in the absence of either ATP (see Fig. 1) or MgCl₂, nor if the ATP was replaced by 5 mM-GTP or 5 mM-ADP (results not shown). The inactivation could largely be prevented by the presence of the inhibitor protein or the omission of cyclic AMP-dependent protein kinase (Fig. 1). These results strongly suggest that glycerol phosphate acyltransferase can be inactivated by a phosphorylation reaction catalysed by added cyclic AMP-dependent protein kinase. The slight inactivation observed in the absence of added cyclic AMP-dependent protein kinase and the presence of the inhibitor protein might suggest that small amounts of a cyclic AMP-independent inactivating factor are present in the microsomal fraction.

Attempts were made to reverse the inactivation of glycerol phosphate acyltransferase. The enzyme

Fig. 1. *Inactivation of glycerol phosphate acyltransferase*
Rat adipose-tissue microsomal fraction was incubated in the standard conditions with the following variations: ●, none; □, ATP omitted; ○, inhibitor protein added; ▲, cyclic AMP-dependent protein kinase omitted and inhibitor protein added. Activities are expressed as specific activities in nmol/min per mg of protein.

was inactivated and dialysed as described in the Experimental section and then incubated under conditions that might be expected to promote dephosphorylation. No increase in activity was observed when the enzyme was incubated in the presence of Mg^{2+} cations or Mn^{2+} cations alone, but the addition of alkaline phosphatase to give a final concentration of 0.1 mg/ml resulted in a rapid reactivation of the enzyme (Fig. 2). The specific activity of the glycerol phosphate acyltransferase reached 75–80% of the value before inactivation. In a control experiment microsomal fractions were treated with cyclic AMP-dependent protein kinase, but no ATP, and then dialysed against 50mM-Tris/HCl, pH 8.5, containing 1mM-EDTA and 1mM-dithiothreitol. The glycerol phosphate acyltransferase was not inactivated and the subsequent addition of alkaline phosphatase had no effect on its activity (Fig. 2).

These results support the idea that glycerol phosphate acyltransferase can be controlled by a phosphorylation–dephosphorylation mechanism and they suggest that an enzyme capable of reactivating the acyltransferase should exist in adipose tissue. However, in a reactivation experiment the addition of the post-microsomal supernatant fraction of

Fig. 2. *Reactivation of glycerol phosphate acyltransferase*
Inactivated and dialysed enzyme (see the Experimental section) was incubated with the following additions: ■, 5mM- $MgCl_2$; ○, 5mM- $MnCl_2$; Δ, 5mM- $MgCl_2$ +0.1mg of alkaline phosphatase/ml. Control enzyme (see the text) was also treated with 0.1mg of alkaline phosphatase/ml and 5mM- $MgCl_2$ (●). Activities are expressed as specific activities in nmol/min per mg of protein.

adipose tissue to give a protein concentration of 0.25mg/ml in the presence of 5mM- $MgCl_2$ did not lead to any detectable reactivation. In another experiment the multifunctional protein phosphatase III from rabbit skeletal muscle was used. This enzyme can dephosphorylate phosphorylase *a*, activated phosphorylase kinase and inactivated glycogen synthase (Antoniw *et al.*, 1977). Even at a concentration of 25 units/ml (where 1 unit is the amount of enzyme that liberates 1nmol of phosphate from phosphorylase *a*/min) in the presence of 5mM- $MnCl_2$ phosphatase III did not give any detectable reactivation of glycerol phosphate acyltransferase.

The results presented above demonstrate that glycerol phosphate acyltransferase can be inactivated in a phosphorylation reaction catalysed by cyclic AMP-dependent protein kinase, and this probably accounts for the inactivation of the enzyme in response to adrenaline that was observed by Sooranna & Saggerson (1976). Two points should be made about

these results *in vitro*. First, the total tissue activity and the specific activity of the microsomal glycerol phosphate acyltransferase observed in the present work are of the same order as values reported previously (Daae, 1973; Jamdar & Fallon, 1973; Schlossman & Bell, 1976). However, the tissue activity (about 10 nmol/min per wet wt. of tissue at 30°C) is rather low in relation to the rate of triacylglycerol synthesis in intact tissue. This can reach 0.9 μ mol/min per g wet wt. of tissue at 37°C under optimal conditions, although in unstimulated tissue it is some 10-fold lower (Denton & Randle, 1967). The reason for this discrepancy is not understood. Secondly, it is known that adrenaline increases rather than decreases the rate of esterification in adipose tissue and fat cells (Vaughan & Steinberg, 1963; Rodbell, 1964) and this might throw doubt on the relevance of the observations reported in the present paper. However, this stimulation of esterification probably results from the greatly increased supply of non-esterified fatty acids under these conditions and is not necessarily incompatible with an inactivation of glycerol phosphate acyltransferase.

The inactivation of glycerol phosphate acyltransferase can be reversed by alkaline phosphatase in what is presumably a dephosphorylation reaction, although the possibility that it involves cleavage of the enzyme by a proteinase contaminating the phosphatase cannot be excluded. No fraction of adipose tissue capable of reversing the inactivation has yet been identified and protein phosphatase III from rabbit skeletal muscle is also unable to reactivate the enzyme. This is in contrast with the activation of hormone-sensitive lipase by cyclic AMP-dependent protein kinase, which can be reversed by an endogenous phosphatase or by liver phosphorylase phosphatase (Severson *et al.*, 1977). Conditions in which glycerol phosphate acyltransferase can be reactivated by an adipose-tissue phosphatase still require identification.

The results presented in the present paper indicate that glycerol phosphate acyltransferase, like hormone-sensitive lipase (Steinberg, 1976), can be controlled by a phosphorylation-dephosphorylation mechanism. They represent the first direct evidence that such mechanisms are involved in the regulation of esterification. This finding increases the analogies that can be drawn between the control systems involved in triacylglycerol and glycogen metabolism. Glycerol phosphate acyltransferase may now, however, be the only point at which triacylglycerol synthesis is controlled hormonally. It has been reported that insulin can increase the activity of fatty acyl-CoA ligase (Jason *et al.*, 1976) and that noradrenaline can cause inactivation of phosphatidate phosphohydro-

lase (Cheng & Saggerson, 1978). Phosphorylation mechanisms may also be involved in these systems.

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