Citrate and the Regulation of Adipose-Tissue Phosphofructokinase

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1. Methods are described for the extraction, partial purification and assay of phosphofructokinase in rat epididymal adipose tissue. 2. The enzyme was inhibited by ATP at concentrations above 20μM and by citrate; the enzyme was activated by ADP, AMP, 3',5'-(cyclic)-AMP, phosphate and sulphate. 3. The enzyme lost activity on incubation at 25° or during chromatography on DEAE-cellulose unless fluoride at a concentration of 4mM was present. 4. The significance of these results in relation to the role of glycolysis and citrate in lipogenesis is discussed.

In rat epididymal adipose tissue the concentration of citrate was increased by adrenaline in vitro and also by alloxan-diabetes (Denton, Yorke & Randle, 1966). In other rat tissues such as heart (Garland, Randle & Newsholme, 1963; Pogson & Randle, 1966) or liver (Underwood & Newsholme, 1965) citrate is an inhibitor of PFK* (ATP-α-fructose 6-phosphate 1-phosphotransferase: EC 2.7.1.11). Moreover, in rat heart PFK is inhibited and the glycolysis rate diminished when the citrate concentration is increased by the respiration of fatty acids or ketone bodies or pyruvate (Newsholme & Randle, 1964). However, the rate of glycolysis in rat epididymal adipose tissue is accelerated by adrenaline (Flatt & Ball, 1964) in spite of the increase in citrate concentration observed by Denton et al. (1966). It seemed important therefore to ascertain whether adipose-tissue PFK was inhibited by citrate and to investigate activation and inhibition by other metabolites that have an influence on the PFK from other rat tissues (adenine nucleotides, phosphate, sulphate).

In adipose tissue glycolysis has a major synthetic role through the provision of acetyl-CoA and \( \text{L-glycerol 3-phosphate} \) for lipogenesis. Moreover, citrate is believed to be of importance in lipogenesis in three ways. Acetyl-CoA formation from pyruvate is believed to occur within the mitochondrion, whereas lipogenesis is thought to occur extramitochondrially. One mechanism proposed for acetyl transfer from intramitochondrial to extramitochondrial compartments involves the conversion of acetyl-CoA into citrate within the mitochondrion catalysed by citrate synthase (EC 4.1.3.7) followed by the extrusion of citrate and breakdown extramitochondrially to acetyl-CoA catalysed by citrate-cleavage enzyme (EC 4.1.3.8) (see Bartley, Abraham & Chaikoff, 1965; Kornacker & Ball, 1965). Moreover, Kornacker & Ball (1965) have proposed that citrate is also involved in NADPH generation for lipogenesis and Matsushima, Matsue & Lymen (1964) find that citrate is an activator of acetyl-CoA carboxylase (EC 6.4.1.2). If citrate is an inhibitor of adipose-tissue PFK and glycolysis this effect could be in conflict with its role in lipogenesis. No such conflict is likely in muscles, where lipogenesis is poorly developed and where a major role for glycolysis is the synthesis of ATP and provision of respiratory fuel. These considerations also suggested the need for an investigation of the sensitivity of adipose-tissue PFK to inhibition by citrate. A preliminary account of some of these findings has been published (Denton & Randle, 1965).

MATERIALS

Rats. Epididymal fat pads were obtained from male albino rats (100-150g.) fed on a stock laboratory diet (Short & Parkes, 1949).

Chemicals and enzymes. Enzymes and other biochemical reagents were obtained from C. F. Boehringer and Soehne G.m.b.H. (through Courtin and Warner Ltd., Lewes, Sussex) except for 3',5'-(cyclic)-AMP (from P. L. Biochemicals Inc., Milwaukee, Wis., U.S.A.). Bovine plasma albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, DEAE-cellulose from Whatman and Sephadex G-25 from Pharmacia (Uppsala, Sweden). Other chemicals were of the purest grade obtainable.

METHODS AND PROCEDURE

Partial purification of PFK

Preparation of crude extract. Fat pads (10-20) were excised from rats killed by decapitation, washed briefly in 50mM-tris-acetate buffer, pH7.5, containing NaF (4mM), blotted lightly and dispersed in 2 vol. of the same buffer at
0° in a hand-operated glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 38000 g for 1 hr. at 4° and the clear aqueous layer removed with a syringe. The activity of the crude extract measured under the conditions given below under ‘Expression of results’ was 0.25 unit/g. wet wt. of fat pad.

DEAE-cellulose fractionation. The procedure was based on that devised for rat-heart PFK by Mr C. I. Pogson, to whom we are indebted for details. A fast-flowing column (4.0 cm. × 2.2 cm.) of DEAE-cellulose was prepared and equilibrated with 50 mM-tris-acetate buffer, pH 7.5, containing NaF (4 mM). The crude extract was run in and elution performed with tris-acetate buffers, pH 7.5, containing NaF (4 mM), of the molarities stated as follows: 50 mM (20 ml.), then 100 mM (10 ml.), then 200 mM (10 ml.), 300 mM (10 ml.) and 400 mM (20 ml.). PFK activity was eluted by 300 mM-tris-acetate buffer. Approx. 70% of the enzyme activity was eluted. The enzyme could not be recovered in high yield unless fluoride was present. The PFK preparation had a maximum activity of 0.5–0.75 unit/mg. (approx. tenfold purification). NADH2-oxidase and adenine-triphosphatase activities were about 1% of the PFK activity. Adenylate kinase was not tested for; it is eluted from DEAE-cellulose by 0.1 M-tris-acetate buffer. Activity was lost on standing at 0° at the rate of 1–2%/hr. and the enzyme was freshly prepared each day.

Assay of PFK activity

The activity was measured enzymically by converting fructose 1,6-diphosphate into L-glycerol 3-phosphate, the resulting disappearance of NADH2 being followed either at 340 μM in a 1 cm. light-path in a Hilger spectrophotometer with Gilford kinetic recording attachment or at 366 μM in a 4 cm. light-path in an Eppendorf photometer fitted with a recorder. Fructose 6-phosphate was generated in situ with glucose 6-phosphate and phosphogluco isomerase. The reaction medium contained (final concentrations): imidazole buffer, pH 7.4 (20 mM), bovine plasma albumin (0.01%), MgCl2 (5 mM), potassium acetate (150 mM), NADH2 (1 mM), together with glucose 6-phosphate isomerase (10 μg/ml.), aldolase (30 μg/ml.), triose phosphate isomerase (1 μg/ml.) and α-glycerophosphate dehydrogenase (10 μg/ml.). The enzymes in the assay were mixed in the correct proportions as the aq. (NH4)2SO4 suspensions dissolved in the minimum of imidazole buffer, pH 7.4 (20 mM), to obtain a clear solution and (NH4)2SO4 was removed on a Sephadex G-25 column.

The following procedure was adopted for each assay. To the reaction medium (2 ml.) was added an appropriate volume of the PFK preparation (usually 100 μl.) together with the desired amounts of ATP, ADP, AMP, 3',5'- (cyclic)-AMP, K2SO4 or (NH4)2SO4, potassium phosphate and potassium citrate, all in imidazole-MgCl2-potassium acetate buffer, pH 7.4, to bring the total volume in the cuvette to 2.4 ml. After 1–2 min., 0.1 ml. of 25 mM-glucose 6-phosphate, which had been allowed to equilibrate with the assay enzymes at room temperature, was added. After an initial lag period of less than 1 min. the rate was linear (to within 5%) for at least 5 min. Rates were always calculated as the mean from the second to the fifth minute.

Expression of results

All activities found are expressed in terms of the maximum activity of the particular preparation of PFK. The latter was measured in medium containing ATP (2 mM), glucose 6-phosphate (1 mM) and (NH4)2SO4 (20 mM). Under these conditions reaction velocity and enzyme concentration were linearly related.

RESULTS

Inhibition of PFK by ATP or citrate. Fig. 1 shows the relationship between enzyme activity and ATP concentration. Increasing the concentration of ATP up to 20 μM increased the velocity of the reaction but increasing the concentrations of ATP above 20 μM diminished the enzyme activity. These measurements, which were made with 0.3 mM-fructose 6-phosphate, also show the low activity of adipose-tissue PFK in the absence of activators, especially of sulphate.

Fig. 2 shows the inhibitory effect of citrate measured with 0.2 mM-ATP and 5 mM-sulphate and with 2 mM-ATP and 10 mM-sulphate. Under these conditions citrate inhibition was seen at concentrations between 2 and 8 mM. In these experiments the concentration of the sulphate employed as an
activator was just sufficient to yield maximum activity of the enzyme. Other experiments showed that the sensitivity of adipose-tissue PFK to citrate inhibition is markedly increased at lower concentrations of sulphate. Thus with 0.2mM-ATP and 2mM-sulphate citrate at a concentration of 2mM results in 75% inhibition of PFK activity.

Activation of PFK by 3',5'-(cyclic)-AMP, ADP, AMP, phosphate and sulphate. Fig. 3 compares the prowess as activators of PFK of 3',5'-(cyclic)-AMP, ADP, AMP, phosphate and sulphate, measured with 0.2mM-ATP and 0.3mM-fructose 6-phosphate. The decreasing order of effectiveness was 3',5'-(cyclic)-AMP, AMP, phosphate and sulphate. The effect of ADP was complicated in that it was almost as effective as AMP at concentrations below 1mM but at higher concentrations it appeared to become inhibitory. Reciprocal plots of activator concentration and reaction velocity were curvilinear and accurate evaluation of the $K_a$ for activation was not possible. Activation by sulphate was observed both with the ammonium and potassium salts; NH$_4^+$ ions (tested as ammonium acetate) had no effect.

Fig. 4 shows the activating effects of 3',5'-(cyclic)-AMP, ADP and AMP on PFK measured with 0.2mM-ATP, 2mM-citrate and 2mM-sulphate. The decreasing order of effectiveness was 3',5'-(cyclic)-AMP, AMP and ADP. PFK was especially sensitive to 3',5'-(cyclic)-AMP under these conditions and 2μM-3',5'-(cyclic)-AMP trebled the reaction velocity.

Inactivation of adipose-tissue PFK and protection by fluoride. Adipose-tissue PFK was found to lose activity on incubation and this loss of activity could be prevented by fluoride. In a typical experiment adipose tissue was extracted with 50mM-tris-0.1mM-EDTA-mM-mercaptoethanol, pH 7.5, and the extract incubated at 25° for 30 and 60min. in the presence and absence of 4mM-fluoride. The initial activity of the extract was 0.07 unit/ml. In the absence of fluoride the activity fell on incubation to 40% of the initial value at 30min. and to 20% at 60min. In the presence of fluoride activity was 80% of the initial activity at 30 and 60min.

DISCUSSION

The present studies were undertaken to ascertain in particular whether adipose-tissue PFK is sensitive to inhibition by citrate, for reasons given in detail in the introduction. These studies have shown that the enzyme is sensitive to inhibition by citrate and that in this and other respects (inhibition by ATP, and activation by ADP, AMP, 3',5'-(cyclic)-AMP, phosphate and sulphate) its behaviour is qualitatively similar to that of PFK from other animal tissues (e.g. see Passoneau & Lowry, 1964). The great sensitivity of the enzyme to activation by 3',5'-(cyclic)-AMP may explain the accelerated glycolysis and flow through PFK induced in adipose tissue by adrenaline in spite of the fact that the hormone increases the concentration of citrate. The concentration of 3',5'-(cyclic)-AMP in adipose tissue incubated with adrenaline (see Butcher, Ho, Meng & Sutherland, 1965) would appear to be high enough to activate PFK.

The possibility that adipose tissue may contain two interconvertible forms of PFK, one of which is insensitive to inhibition by citrate, is difficult to exclude. Viñuela, Salas, Salas & Soló (1964) have obtained evidence for the existence in yeast of two forms of PFK, one of which is insensitive to inhibition by ATP. The conditions that we have used for the extraction of adipose tissue (use of fluoride) are those that yielded a form of the yeast enzyme insensitive to inhibition by ATP in the experiments.
of Viñuela et al. (1964). The adipose-tissue enzyme was sensitive to inhibition both by ATP and citrate under these conditions. The only evidence that we have obtained for the possible existence of a second form of the enzyme was in experiments in which the enzyme was extracted and incubated at 25° in the absence of fluoride. This treatment did not, however, change the sensitivity of the enzyme to inhibition by citrate or ATP. Rather it resulted in substantial loss of enzyme activity (up to 80% loss). In this respect adipose-tissue PFK resembles more closely guinea-pig heart PFK (Mansour, 1965) and not yeast PFK.

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REFERENCES


