Adipose-tissue phosphofructokinase

Rapid purification and regulation by phosphorylation *in vitro*

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A new procedure for the purification of phosphofructokinase using Blue Dextran–Sephadex is described. This allowed an approx. 1000-fold purification of phosphofructokinase from rat white and brown adipose tissue to be achieved in essentially a single step. The purified enzymes from both tissues were found to exhibit hyperbolic kinetics with fructose 6-phosphate, to be inhibited by ATP and citrate, and to be activated by 5'-AMP, phosphate and fructose 2,6-bisphosphate. The enzymes were phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase, and phosphorylation was found to be associated with increases in activity when the enzymes were assayed under appropriate sub-optimal conditions. In particular, the phosphorylated enzymes exhibited less inhibition by ATP and the white-adipose-tissue enzyme was more sensitive to activation by fructose 2,6-bisphosphate. It is suggested that an increase in the cytoplasmic concentration of cyclic AMP in tissues other than liver may result in an increase in glycolysis through the phosphorylation of phosphofructokinase by cyclic AMP-dependent protein kinase.

INTRODUCTION

Increases in cyclic AMP concentrations in liver cells, for example after exposure of the cells to glucagon, are associated with the inhibition of glycolysis and activation of gluconeogenesis (Hers & Hue, 1983; Pilkis *et al.*, 1978). It is now well established that these changes are in part brought about through a diminution in the concentration of fructose 2,6-bisphosphate, which is not only a potent activator of phosphofructokinase but also an inhibitor of fructose-1,6-bisphosphatase (Van Schaftingen *et al.*, 1980*a,b*, 1981; Hers & Van Schaftingen, 1982; Pilkis *et al.*, 1982). The diminution in fructose 2,6-bisphosphate concentration is the result of changes in the activity of fructose-6-phosphate-2-kinase/fructose-2,6-bisphosphatase, which follow the phosphorylation of this bifunctional enzyme by cyclic AMP-dependent protein kinase (El-Maghrabi *et al.*, 1982*a,b*; Furuya *et al.*, 1982*a,b*; Murray *et al.*, 1984). However, in most other cells, including white and brown fat-cells, an increase in cyclic AMP would be expected to be associated with an increase in the rate of glycolysis (rather than the decrease found in liver), and thus a different mechanism or mechanisms which allows stimulation of phosphofructokinase must operate under these conditions.

There have been several reports of increased phosphofructokinase activity assayed at low concentrations of fructose 6-phosphate in crude extracts of muscle previously exposed to catecholamines (Mansour, 1972; Beitner *et al.*, 1978; Clark & Patten, 1984). In the heart, the effects appear to be exerted primarily through α -receptors (Clark & Patten, 1984), and are not therefore cyclic AMP-dependent.

Exposure of white fat-cells from rat epididymal adipose tissue to adrenaline causes an increase in glycolysis (Katz *et al.*, 1966; Halperin & Denton, 1969; Saggerson & Greenbaum, 1970) and also results in

persistent changes in phosphofructokinase activity measured in subsequently prepared extracts. However, the changes are apparently rather conflicting, since they are a combination of an increase in the apparent affinity of phosphofructokinase for fructose 6-phosphate together with a small decrease in $V_{\max}$ (Sooranna & Saggerson, 1982; Lederer & Hers, 1984). Since no changes in the concentration of fructose 2,6-bisphosphate were observed under these conditions (Lederer & Hers, 1984; Rider & Hue, 1985), it was concluded that the changes in activity may involve alterations in the binding of another tightly bound ligand, perhaps fatty acids. However, an alternative hypothesis would be that the persistent changes in kinetic properties were the result, at least in part, of an increase in phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. Phosphofructokinase from a number of different sources has been shown to be a substrate for cyclic AMP-dependent protein kinase, including liver (Furuya & Uyeda, 1980; Pilkis *et al.*, 1982), skeletal muscle (Riquelme *et al.*, 1978*a,b*; Kemp *et al.*, 1981) and kidney, brain and red cells (Mendicino *et al.*, 1978; Marcus *et al.*, 1979; Lagrange *et al.*, 1980), but the effect of phosphorylation on catalytic activity has been small and rather conflicting. One possible explanation for this might be partial proteolysis of the enzyme during the lengthy procedures used for purification.

In this paper, we describe a new procedure which allows about 1000-fold purification of phosphofructokinase from white and brown adipose tissue in essentially a single step. We also show for the first time that the purified enzymes from both tissues are substrates for cyclic AMP-dependent protein kinase and that phosphorylation results in modest increases in activity when the enzymes are assayed under appropriate conditions. In the accompanying paper, we present direct evidence for the increased phosphorylation of phosphofructokinase in rat

epididymal adipose tissue exposed to isoprenaline and adrenaline and that this may be associated with activation of the enzyme (Sale & Denton, 1985).

EXPERIMENTAL

Animals

Epididymal white adipose tissue and interscapular brown adipose tissue were from male albino Wistar rats (150–200 g) allowed free access to water and a stock laboratory diet (modified 41B; Oxoid, Basingstoke, Hants., U.K.) and housed at 22–25 °C.

Materials

Chemicals were analytical grade from BDH Chemicals, Poole, Dorset, U.K. Biochemical reagents and enzymes were from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K.

Sephacrose 4B (cross-linked) and Blue Dextran were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and used to prepare Blue Dextran–Sephacrose as described by Ryan & Vestling (1974). The resin was washed extensively with 5 mM-potassium phosphate/5 mM-MgSO₄/1 mM-EDTA/0.5 mM-dithiothreitol/0.4 M-KCl, pH 7.1, after each use and stored at 4 °C in distilled water containing 0.02% Na₂S₂O₃.

Samples of fructose 2,6-bisphosphate were kindly given by Dr. E. Van Schaftingen and Professor H.-G. Hers (Université de Louvain, Brussels, Belgium) and by Dr. S. Pilkis (Vanderbilt School of Medicine, Nashville, TN, U.S.A.). The catalytic subunit of cyclic AMP-dependent protein kinase freshly prepared from ox heart was kindly given by Dr. Mark Seville and Dr. Paul England of this Department.

Extraction and purification of phosphofructokinase from white adipose tissue

Epididymal fat-pads (6–20 g in total) were incubated for 30 min in 5 ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 1.5 mM-CaCl₂ and 5 mM-glucose/g. Tissue was blotted and approx. 1 g samples were immediately extracted at 0 °C in 2 ml of 5 mM-potassium phosphate/5 mM-MgSO₄/1 mM-EDTA/0.5 mM-dithiothreitol, pH 7.1 (buffer A), by use of a Polytron PT-20 homogenizer (speed 5 for 15 s). Extracts were combined and centrifuged at about 1000 *g* for 30 s. This and all subsequent steps were carried out at 0–5 °C. The resulting infranatant between the floating fat plug and pelleted cell debris was removed by syringe and centrifuged at about 30 000 *g* for 30–45 min. The supernatant was applied to a small Blue Dextran–Sephacrose 4B column (2–5 ml), which had previously been equilibrated by passing through at least 200 ml of buffer A. After application of the supernatant (flow rate 0.5 ml/min), the column was washed with buffer A (flow rate 1 ml/min) until the eluate was essentially free of protein (Fig. 1). This was usually about 150 ml. The column was then washed at the same flow rate with successively 100 ml of buffer A with 2 mM-AMP, about 50 ml of buffer A without addition and 100 ml of buffer A with 5 mM-fructose 6-phosphate. Finally, phosphofructokinase was eluted from the column as a single sharp peak by buffer A containing both 2 mM-AMP and 5 mM-fructose 6-phosphate. Column fractions (3 ml) containing appreciable phosphofructokinase activity

were pooled (total volume 18–24 ml) and concentrated to approx. 0.5 ml by dialysis at 4 °C against a saturated solution of sucrose. The dialysis tubing was re-tied to eliminate slack and the sucrose was removed by dialysis against at least 500 vol. of buffer A. In some experiments, it was found necessary to repeat the concentration procedure to reduce the volume to 0.5 ml. The final preparation was stored in small samples at –15 °C. No loss of activity was found during storage for up to 4 months.

Extraction and purification of phosphofructokinase from brown adipose tissue

Details were as for white tissue, with the following changes. Interscapular brown tissue was removed from animals under barbitone anaesthesia. Because brown adipose tissue contains more phosphofructokinase than does white adipose tissue (on a wet-weight basis), 10 ml Blue Dextran–Sephacrose columns were routinely used for the purification of phosphofructokinase from 6–30 g of tissue (obtained from 10–50 rats).

Assay of phosphofructokinase

Activity was measured at 30 °C by following the production of fructose 1,6-bisphosphate. Samples containing up to 5 ml of phosphofructokinase were added to 1.5 ml of assay buffer (50 mM-Hepes/1 mM-EDTA/1 mM-NH₄Cl/6.5 mM-MgCl₂/2.5 mM-dithiothreitol/0.16 mM-NADH/100 mM-KCl, usually pH 7.1) containing the coupling enzymes aldolase (0.3 unit/ml) triosephosphate isomerase (1.8 units/ml) and L-glycerol-3-phosphate dehydrogenase (0.3 unit/ml) and further additions as given in the text or Figure legends. The coupling enzymes were desalted before use by overnight dialysis at 4 °C against at least 200 vol. of 50 mM-Hepes buffer, pH 7.1. For the assay of maximal activity the additions were 5 mM-potassium phosphate, 5 mM-fructose 6-phosphate and 2 mM-AMP, and the reaction was initiated by the addition of 0.5 mM-ATP. The rate of NADH oxidation was monitored in a recording spectrophotometer at 340 nm. All assays were linear after a short (about 1 min) lag period and were proportional to the volume of sample used.

Preparation of chicken antiserum to brown-adipose-tissue phosphofructokinase

Antisera were raised in 6-week-old pullets as described by Hopkirk & Bloxham (1979). Chickens were given subcutaneous injections of 50 µg of purified brown-adipose-tissue phosphofructokinase (48 units/mg of protein) in 1 ml of Freund's complete adjuvant at weekly intervals for 3 weeks and then a final injection after a further 2 weeks. Blood was collected 2 weeks after this injection when the maximum titre was achieved; serum was prepared and small samples were stored at –15 °C. The antiserum was able to precipitate phosphofructokinase activity from extracts of both white and brown adipose tissue as well as from purified preparations. Incubations were carried out in 20 mM-Tris/HCl/0.25 M-sucrose/5 mM-EGTA/7.5 mM-GSH, pH 7.5, at 4 °C for 1 h, and immunoprecipitates were sedimented by centrifugation at room temperature in a Beckman Airfuge at approx. 100 000 *g* for 10 min. Under these conditions the antiserum had a titre of 10 and 15 units/ml against white- and brown-adipose-tissue phosphofructokinase respectively. An unusual feature of the antiserum was that it caused only a small diminution (less than 20%) in the

maximum activity of white- or brown-adipose-tissue phosphofructokinase. Measurements of phosphofructokinase activity could therefore be used to follow the fate of the enzyme during immunoprecipitation.

Expression of results

A unit of enzyme activity was taken as the amount that catalysed the formation of products at the rate of $1 \mu\text{mol/min}$ at 30°C .

Enzyme kinetic constants were calculated by fitting to the following equations: for substrate $v = V_{\text{max}}/[1 + (K_m/s)^n]$, for inhibitor $v = V_o/[1 + I/K_i]^n$, and for an activator $v = [V_{\text{max}}/(1 + K_a/A)] + V_o$, where V_{max} is maximal velocity, V_o is the rate in the absence of added activator or inhibitor, and s , I and A are the concentrations of substrate, inhibitor and activator respectively; K_m , K_i and K_a are the concentrations giving half-maximal response. Constants were calculated by using a non-linear least-squares regression program written for a Hewlett-Packard 9821 computer by Dr. Paul England of this Department.

Other methods

Protein was determined either by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, or from the A_{280} . SDS/polyacrylamide-gel electrophoresis was carried out in 1 cm tracks on 8% (w/v)-polyacrylamide slab gels by the method of Laemmli (1970). Protein samples were dissolved in 'SDS-gel buffer' (62.5 mM-Tris/HCl containing 200 mg of sucrose/ml, 0.2 mg of Bromophenol Blue/ml and 100 mM-2-mercaptoethanol, pH 7.4) and heated at 100°C for 5 min. Electrophoresis was performed at 20°C for about 3 h at 30–35 mA. Gels were washed with 20% (v/v) trichloroacetic acid in 50% (v/v) methanol and then stained with Coomassie Brilliant Blue. After destaining, gels were washed in 10% (v/v) glycerol and dried on to Cellophane. Radioautographs were obtained by exposing the gel to pre-flashed Kodak X-omat S film at -80°C for 1–14 days within cassettes containing Dupont Cronax intensifier screens. ^{32}P associated with appropriate protein bands was determined by incubating appropriate gel pieces for 2 h at 80°C in sealed scintillation vials containing 400 μl of H_2O_2 ; the vials were then opened, the contents evaporated to dryness, and radioactivity was determined after addition of 1 mM-HCl (200 μl) and 5 ml of scintillation fluid [naphthalene (80 g/l) and 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxo-3,4-diazole (6g/l) in toluene/methoxyethanol (3:2, v/v)].

RESULTS

Use of affinity elution from Blue Dextran–Sephadex to purify adipose-tissue phosphofructokinase

Affinity elution from Blue Dextran–Sephadex or Cibacron Blue–Sephadex has been used by others to purify phosphofructokinase from a variety of sources, including mammalian liver (Mendicino *et al.*, 1975; Kagimoto & Uyeda, 1979), yeast (Hofmann & Kopperschlager, 1982), red cells (Layzer, 1975) and bacteria (Kotlarz & Blue, 1982; Fordyce *et al.*, 1982). In these procedures elution was effected with buffers containing added ATP or ATP plus fructose 1,6-bisphosphate, and 5–50-fold increase in specific activity was achieved. However, we found that much more specific elution of phosphofructokinase could be achieved by elution of the

Blue Dextran–Sephadex column with 5 mM-potassium phosphate buffer containing both 5 mM-fructose 6-phosphate and 2 mM-AMP. Although the presence of either 5 mM-fructose 6-phosphate or 2 mM-AMP caused the elution of other proteins, the combination of fructose 6-phosphate and AMP was required to cause the elution of phosphofructokinase under the conditions employed. Thus, by utilizing column washes with buffer containing first only AMP and then only fructose 6-phosphate before elution of phosphofructokinase, it was possible to achieve an approx. 1000-fold purification of the enzyme from supernatant fractions of both white and brown adipose tissue (Fig. 1). The entire procedure was complete within one working day, except for the final dialysis and concentration steps.

Typical examples of the use of procedure to purify phosphofructokinase from white adipose tissue are given in Fig. 1 and Table 1. The amount of phosphofructokinase in white adipose tissue is about $4 \mu\text{g/g}$ wet wt., assuming a specific activity of 80–100 units/mg. The overall yield in 14 different preparations was $44 \pm 5.5\%$ (mean $\pm$ S.E.M.), which was considered highly satisfactory in view of the small amount of protein. The final specific activity was 35.4 ± 7 units/mg of protein, which represented a purification of 1480 ± 220 -fold (mean $\pm$ S.E.M. for three separate preparations). The major protein in the preparations migrated on SDS/polyacrylamide-gel electrophoresis with an apparent subunit M_r of 78000, slightly greater than that of a commercial preparation of rabbit skeletal-muscle phosphofructokinase. Similar subunit M_r values as determined by SDS/polyacrylamide-gel electrophoresis have been reported for phosphofructokinase from other tissues (see Uyeda, 1979). On the basis of the Coomassie Blue staining of the proteins in white-adipose-tissue phosphofructokinase preparations separated by SDS/polyacrylamide-gel electrophoresis, phosphofructokinase represented about 40% of the protein in the preparations. Making allowance for this purity, the specific activity of pure white-adipose-tissue phosphofructokinase would be 80–90 units/mg of protein, which is close to the values found for phosphofructokinase from other mammalian tissues (Uyeda, 1979).

An example of a typical purification from brown adipose tissue is given in Table 1. Yield through the purification procedure ($63.8 \pm 3.3\%$ for four preparations) was greater than for white adipose tissue, probably owing to the greater amount of phosphofructokinase. The final preparations were very similar to those from white adipose tissue with respect to specific activity (mean value $\pm$ S.E.M. of 42 ± 3.5 units/mg for three preparations), apparent subunit M_r on SDS/polyacrylamide-gel electrophoresis of 78000 and purity (40–50%) as judged by Coomassie Blue staining.

Kinetic properties of phosphofructokinase purified from white and brown adipose tissue

Preparations of both white- and brown-adipose-tissue phosphofructokinase were found to have the kinetic properties characteristic of phosphofructokinase from other mammalian tissues. Earlier studies on the properties of rat epididymal white adipose tissue employed either crude extracts (Sooranna & Saggerson, 1982; Lederer & Hers, 1984) or a modest 10-fold purification by DEAE-cellulose chromatography (Denton & Randle, 1966). However, it is difficult, if not impossible, to carry

Fig. 1. Typical separation of phosphofructokinase from an extract of rat epididymal white adipose tissue by using Blue Dextran–Sephadex

Supernatant fraction prepared from 18 g wet wt. of pads was applied to a 5 ml column of Blue Dextran–Sephadex equilibrated with 5 mM-potassium phosphate buffer, pH 7.1, containing 5 mM-MgCl₂, 1 mM-EGTA and 0.5 mM-dithiothreitol. The column was then eluted sequentially with the same buffer with or without added 2 mM-AMP or 5 mM-fructose 6-phosphate (F6P) as indicated. Fractions (3.3 ml) were collected and assayed for phosphofructokinase (●). ○, A₂₈₀ (protein). See the Experimental section for further details.

Table 1. Purification of phosphofructokinase from rat epididymal white adipose tissue and interscapular brown adipose tissue

Data from typical preparations are shown.

Step	Volume (ml)	Phosphofructokinase activity		Purification (fold)	Yield (1%)
		Total (units)	Specific activity (units/mg)		
(a) White adipose tissue (18 g wet wt. of tissue)					
Initial 30000 g supernatant	38	5.12	0.02	1.0	100
Blue Dextran–Sephadex	17	2.80	—	—	55
Dialysis and concentration	1.4	1.84	34.2	1630	36
(b) Brown adipose tissue (25 g wet wt. of tissue)					
Initial 30000 g supernatant	25	88	0.083	1.0	100
Blue Dextran–Sephadex	36	87	—	—	98.5
Dialysis and concentration	4.6	59	42.0	506	67.2

out precise studies on kinetic properties in whole-cell extracts, or even on partially purified enzyme, because of (i) the continuous production of the activators AMP, ADP and P_i owing to the presence of ATPase and adenylate kinase activity, (ii) the conversion of fructose 6-phosphate by phosphoglucosmutase, and (iii) the possibility of tightly bound effectors such as fructose 2,6-bisphosphate and fatty acids (Lederer & Hers, 1984) remaining bound to differing extents to the enzyme. The properties of phosphofructokinase from brown adipose tissue have not previously been reported.

The principal features found with the purified enzyme from both brown and white adipose tissue were that sigmoidal kinetics (Hill coefficient close to 2) were

exhibited with respect to one substrate, fructose 6-phosphate (Fig. 4*a*), whereas increasing inhibition was found at higher concentrations of the other substrate, ATP (Fig. 3), especially with the brown-adipose-tissue enzyme. This inhibition by ATP was diminished at higher concentrations of fructose 6-phosphate and by the presence of a number of activators, including 5'-AMP, phosphate (results not shown) and, as illustrated in Fig. 4*b*), fructose 2,6-bisphosphate. In the presence of 1.0 mM-ATP and 0.2 mM-fructose 6-phosphate the apparent K_a for activation by fructose 2,6-bisphosphate was $0.7 \pm 0.15 \mu\text{M}$ for the white-adipose-tissue enzyme and $0.5 \pm 0.07 \mu\text{M}$ for the brown-adipose-tissue enzyme. Results are given for parameter values $\pm$ S.E.M., based on

observations on six white- and two brown-adipose-tissue preparations. It should be noted that the brown-adipose-tissue enzyme showed co-operative kinetics (Hill coefficient = 2.1), whereas this was not evident with the white-adipose-tissue enzyme. The enzymes were also inhibited by citrate. For example, it was found with the brown-adipose-tissue enzyme, assayed under conditions that gave near-maximal activity (1 μ M-fructose 6-phosphate, 1 mM-ATP and 0.1 mM-AMP), that citrate inhibition was non-co-operative and had an apparent K_i of 0.17 ± 0.01 mM (parameter value $\pm$ S.E.M. for 18 degrees of freedom).

Phosphorylation of the phosphofructokinase preparations by the catalytic subunit of cyclic AMP-dependent protein kinase

Preparations of both white- and brown-adipose-tissue phosphofructokinase were found to be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase (Fig. 2). Incorporation of 32 P from [γ - 32 P]ATP was mainly into the M_r -78000 band separated by SDS/polyacrylamide-gel electrophoresis, but incorporation into other proteins in the preparations was also evident. However, only the labelled protein of M_r 78000 was immunoprecipitated with antiserum raised against brown-adipose-tissue phosphofructokinase. Under the conditions used, maximum phosphorylation was observed at 30 min. There was little or no incorporation of 32 P in the absence of added protein kinase.

Estimates of the stoichiometry of 32 P incorporated into phosphofructokinase were made from the 32 P content of the M_r -78000 band of the immunoprecipitates, the specific radioactivity of the ATP and the amount of phosphofructokinase calculated from catalytic activity in the immunoprecipitates (see the Experimental section for further details). Values obtained were 0.86 ± 0.26 (6) and 0.60 ± 0.13 (5) mol of 32 P incorporated/mol of enzyme tetramer for preparations of enzymes from white and brown adipose tissue respectively. These may be low estimates, as no allowance could be made for any incomplete recovery of enzyme through polyacrylamide-gel electrophoresis. Because of the presence of serum-derived proteins in the immunoprecipitates, it was not possible to determine the amount of phosphofructokinase (which would have been in the range 1.5–3.0 μ g) from Coomassie Blue staining of the gels.

Effects of phosphorylation on the activity of phosphofructokinase

Although the maximal extent of phosphorylation of phosphofructokinase from both white and brown adipose tissue by the catalytic subunit of cyclic AMP-dependent protein kinase appeared to be less than 1 mol/mol of subunit under the conditions employed, changes in kinetic properties were nevertheless evident, and these are illustrated in Figs. 3 and 4.

Phosphorylation did not alter the maximum activity measured at pH 7.1 in the presence of 5 mM-potassium phosphate, 5 mM-fructose 6-phosphate, 2 mM-AMP and 0.5 mM-ATP. However, phosphorylation resulted in the activation of the enzyme when assayed under conditions where the activity of the enzyme was less than maximal. In particular, the inhibitory effects of ATP concentrations above 1 mM were consistently less for both the white- and brown-adipose-tissue enzymes after phosphorylation

Fig. 2. Phosphorylation of purified (a) white- and (b) brown-adipose-tissue phosphofructokinase by catalytic subunit of cyclic AMP-dependent protein kinase

Samples of phosphofructokinase (1.5–3.0 μ g) were preincubated for 2 min at 37 °C in 20 mM-sodium phosphate/20 mM-NaF/5 mM-MgCl₂/0.5 mM-EDTA/0.5 mM-dithiothreitol in the absence (track i) or presence (tracks ii and iii) of catalytic subunit of cyclic AMP-dependent protein kinase (0.5 munit) in a total volume of 40 μ l. Additions of [γ - 32 P]ATP (100 μ M; 200 d.p.m./pmol) were then made, and incubations continued for 30 min. Reaction was terminated by addition of 'SDS-gel buffer' either directly (tracks i and ii) or after immunoprecipitation with 15 μ l of antiserum raised against purified rat brown-adipose-tissue phosphofructokinase in the presence of further additions of 10 mM-EDTA/20 mM-sodium phosphate, pH 7.5 (track iii). Proteins were separated by SDS/polyacrylamide-gel electrophoresis on an 8% (w/v) polyacrylamide gel, which was dried, and 32 P-labelled phosphoproteins were detected by autoradiography.

by the catalytic subunit (Fig. 3). The changes evident in Fig. 3 were typical of those found with other white- and brown-adipose-tissue enzyme preparations phosphorylated to a similar extent. For example, with three different preparations of the brown-adipose-tissue enzyme the activity assayed as in Fig. 3 at 1 mM-ATP was 2.2 ± 0.4 -fold (mean $\pm$ S.E.M.) higher after phosphorylation. Incubation of phosphofructokinase preparations with either added ATP alone (Fig. 4) or added catalytic subunit alone (results not shown) had no appreciable effects on catalytic activity, and no effects of phosphorylation on the apparent K_m for fructose 6-phosphate were found when assays were carried out at a non-inhibitory concentration of ATP (Fig. 4).

For the white-adipose-tissue enzyme, but not the brown-adipose-tissue enzyme, phosphorylation was also

Fig. 3. Effects of phosphorylation by catalytic subunit of cyclic AMP-dependent protein kinase on the activity of purified (a) white- and (b) brown-adipose-tissue phosphofructokinase at various concentrations of ATP

Phosphofructokinase was incubated with (○) no additions, (△) $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or (●) catalytic subunit of cyclic AMP-dependent protein kinase plus $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min at 30 °C as described in Fig. 2. Reaction was terminated by addition of 10 mM-EDTA/20 mM-potassium phosphate, pH 7.5. Assays were carried out at pH 7.1 in the presence of 5 mM-potassium phosphate and 1 mM-fructose 6-phosphate.

found to result in a substantial decrease in the apparent K_a for fructose 2,6-bisphosphate (Fig. 4). In the experiment illustrated in Fig. 4, phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase resulted in a decrease in the K_a from $0.58 \pm 0.11 \mu\text{M}$ to $0.30 \pm 0.06 \mu\text{M}$. In another study with a separate enzyme preparation, the K_a was diminished from $0.93 \pm 0.08 \mu\text{M}$ to $0.41 \pm 0.09 \mu\text{M}$ (values are given as parameter $\pm$ S.E.M. for 11–15 degrees of freedom). Incubation of purified

Fig. 4. Effects of phosphorylation by catalytic subunit of cyclic AMP-dependent protein kinase on the activity of purified white-adipose-tissue phosphofructokinase at various concentrations of (a) fructose 6-phosphate and (b) fructose 2,6-bisphosphate

Phosphofructokinase was incubated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ with (●) or without (○) catalytic subunit of cyclic AMP-dependent protein kinase plus $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as in Fig. 3. Assays were carried out at pH 7.1 in the presence of 1 mM-potassium phosphate and 1 mM-ATP. In (b) the concentration of fructose 6-phosphate was 0.2 mM.

white-adipose-tissue phosphofructokinase with added ATP alone or added catalytic subunit alone had no effect.

DISCUSSION

In the present paper we describe a novel technique for the rapid purification of phosphofructokinase from both white and brown adipose tissue in essentially a single step. The final preparations were approx. 40% pure, representing a 500–1500-fold purification. Further purification was not attempted, partly because of the small amount of enzyme available, but mainly because the enzyme preparations were sufficiently pure for the present purposes, including the raising of antisera (see also Sale & Denton, 1985). Preliminary studies showed that the liver enzyme could also be successfully purified by

essentially the same procedure, and it seems likely that the technique could be applied to phosphofructokinase from other sources with the minimum of modification.

The subunit M_r and catalytic properties of phosphofructokinase purified from both white and brown adipose tissue were broadly similar to those found from previous studies on phosphofructokinase purified from other mammalian tissues. In particular, both enzymes are activated by fructose 2,6-bisphosphate at sub-micromolar concentrations and inhibited by citrate.

Phosphofructokinase from both white and brown adipose tissue was phosphorylated by cyclic AMP-dependent protein kinase, but the maximum incorporation of phosphate appeared to be less than 1 mol/mol of tetramer. Phosphorylation of liver and skeletal-muscle enzymes by the same kinase has been reported to approach 4 mol/mol of tetramer (Riquelme *et al.*, 1978*b*; Pilkis *et al.*, 1982). One possible explanation is that the adipose-tissue enzymes may already be partially phosphorylated on purification. This seems most unlikely for the white-adipose-tissue enzyme, since the tissue was preincubated in the absence of hormones before extraction. Under these conditions the extent of phosphorylation appears to be extremely low (Sale & Denton, 1985). However, it is an important possibility for the brown-adipose-tissue enzyme, where preincubation was considered unwise, because of the large derangement of metabolism that occurs on incubation of rat interscapular brown adipose tissue (McCormack & Denton, 1977; McCormack, 1982). In any case, estimates of the extent of phosphorylation in this paper are minimal estimates, since we had to make the assumption that recovery of phosphofructokinase into the M_r -78000 protein band separated by SDS/polyacrylamide gel electrophoresis was 100%, and this is unlikely. It has also to be pointed out that the concentration of phosphofructokinase in the phosphorylation reaction was less than $1\ \mu\text{M}$, because of the small amounts of purified enzyme available. This is very low compared with the reported values of the apparent K_m of cyclic AMP-dependent protein kinase for skeletal-muscle and liver phosphofructokinase of 100–250 μM (Kemp *et al.*, 1981; Pilkis *et al.*, 1982), and this may be the principal reason for the less than stoichiometric incorporation of ^{32}P into white- or brown-adipose-tissue phosphofructokinase in the present studies.

Finally, we present evidence that phosphorylation can lead to activation of phosphofructokinase derived from both white and brown adipose tissues. The activation is mainly characterized by a diminution in the inhibitory effects of ATP and, at least for the white-adipose-tissue enzyme, by a decrease in the apparent K_m for the activator, fructose 2,6-bisphosphate. This is in apparent contrast with the changes that have been reported to be associated with the phosphorylation of the enzyme from liver or skeletal muscle (Riquelme & Kemp, 1980; Furuya & Uyeda, 1980; Foe & Kemp, 1982; Sakakibara & Uyeda, 1983; Kitajima *et al.*, 1983). In these cases, small increases in the inhibitory effect of ATP and an increase in the apparent K_a for fructose 2,6-bisphosphate were found. Our findings suggest that, under conditions of elevated cytoplasmic concentrations of cyclic AMP, increased phosphorylation of phosphofructokinase in both white and brown adipose tissue may occur, and this could increase the activity of phosphofructokinase and hence explain, at least in part, the stimulation of

glycolysis. Direct evidence for this contention is given in the accompanying paper (Sale & Denton, 1985).

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