

Kinase activator protein mediates longer-term effects of starvation on activity of pyruvate dehydrogenase kinase in rat liver mitochondria

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Starvation of rats for 48 h increased the activity of PDH (pyruvate dehydrogenase) kinase 2.2-fold in extracts of liver mitochondria, 2.9-fold in PDH complex partially purified therefrom by fractional precipitation, and 5-fold in PDH complex partially purified by gel filtration on Sephacryl S-300. A protein fraction was separated from PDH complex in extracts of rat liver mitochondria by gel filtration or fractional precipitation, which increased the activity of PDH kinase in rat liver and pig heart PDH complexes. The activity of this protein fraction was increased approx. 2.5-fold by 48 h starvation of rats. With highly purified pig heart PDH complex it was shown that the protein fraction increased the V_{\max} of the PDH kinase reaction 35-fold (fraction from fed rats) or 82-fold (fraction from starved rats); starvation had no effect on the concentration of protein fraction required to give 0.5 V_{\max} . Evidence is given that the increase in PDH kinase activity effected in extracts of liver mitochondria by starvation is due to increased activity of kinase activator protein, which is tightly bound by rat liver PDH complex and not removed by a single gel filtration. With pig heart PDH complex, increased PDH kinase activity was retained after gel filtration of an admixture with kinase activator protein from starved rats, but was restored to the control value by a second gel filtration; the alterations in PDH kinase activity were associated with obvious changes in protein bands in SDS gels.

INTRODUCTION

Mammalian mitochondrial PDH complexes are regulated by reversible phosphorylation catalysed by PDH kinase intrinsic to the complexes and by PDH phosphatase, which is readily separated from PDH complexes by differential centrifugation or gel filtration; phosphorylation is inactivating (Linn *et al.*, 1969*a,b*; Teague *et al.*, 1982; Stepp *et al.*, 1983). There are three sites of phosphorylation in an α -chain of the E1 component (Yeaman *et al.*, 1978; Sugden *et al.*, 1979). Inactivation is almost wholly due to phosphorylation of site 1; relative rates of phosphorylation are sites 1 > 2 > 3 (Sale & Randle, 1981, 1982*a,b*).

The percentage of PDH complex in the active form is decreased by starvation (48 h) or alloxan-diabetes in rat heart, skeletal muscle, liver, adipose tissue and kidney; the decrease ranges from up to 3-fold in liver to 30-fold in heart (for reviews see Wieland, 1983; Randle, 1986). Two mechanisms are known which may decrease the percentage of active PDH complex in starvation and diabetes. Oxidation of fatty acids (and ketone bodies) may activate PDH kinase by increasing mitochondrial ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD⁺]. This is a shorter-term mechanism and may be rapidly reversed in heart muscle and kidney by inhibitors of fatty acid oxidation such as 2-tetradecylglycidate (Caterson *et al.*, 1982). There is a further and longer-term mechanism involving PDH kinase activation, which is not mediated by short-term effects of acetyl-CoA or NADH. This has been shown in mitochondria or in extracts of mitochondria from rat heart, skeletal muscle or mammary gland

(Kerbey *et al.*, 1977; Hutson & Randle, 1978; Baxter & Coore, 1978; Kerbey & Randle, 1981, 1982; Fuller & Randle, 1984). This further mechanism is a stable mechanism which persists through isolation of mitochondria and incubation of mitochondria in the absence of respiratory substrates or with uncouplers of oxidative phosphorylation (to effect conversion of inactive PDH complex into active PDH complex), and through extraction and incubation of mitochondrial extracts.

Rat heart mitochondrial extracts contain a protein (or proteins) (kinase activator protein) which activate PDH kinase; the activity of kinase activator protein is increased by starvation or diabetes (Kerbey & Randle, 1981, 1982; Kerbey *et al.*, 1984). Kinase activator protein may be separated from PDH complex (containing PDH kinase) (M_r approx. 10^7) by precipitation of the latter by centrifugation at 150 000 *g* or by gel filtration on Sephacryl S300. By gel filtration it was found that the activity of PDH kinase intrinsic to the PDH complex and the activity of kinase activator protein were both increased by starvation (Kerbey *et al.*, 1984). It was not clear whether the effect of starvation on intrinsic PDH kinase was an effect of kinase activator protein bound tightly to PDH complex or was due to a further, unknown, mechanism. Further progress was made difficult by the small quantities of PDH complex and kinase activator protein that could be obtained from rat heart.

In the present study methods have been developed for separating PDH complex (with PDH kinase) and kinase activator protein by employing rat liver mitochondria,

Abbreviations used: PDH, E1, pyruvate dehydrogenase (EC 1.2.4.1); E2, dihydrolipoamide acetyltransferase (EC 2.3.1.12); E3, dihydrolipoamide dehydrogenase (EC 1.8.1.4); PDH kinase, pyruvate dehydrogenase (lipoamide) kinase (EC 2.7.1.99).

which are obtainable in much greater quantity. Evidence is given that kinase activator protein is present in liver, that its activity is increased by starvation, that the effect of starvation is on the specific activity of kinase activator protein, that the effect of starvation on PDH kinase intrinsic to PDH complex is mediated by kinase activator protein bound by the complex, and that highly purified pig heart PDH complex (with PDH kinase) may be used to assay and to bind rat liver kinase activator protein.

EXPERIMENTAL

Materials

Details relating to male Wistar rats (source, feeding) and sources of chemicals, biochemicals, [γ - 32 P]ATP, Centriflo cones, Sephacryl, Sephadex and X-ray film are as in Kerbey *et al.* (1979, 1984) and Espinal *et al.* (1986).

Mitochondrial extracts

Rat liver mitochondria were prepared by a modified method in which digitonin was used to disrupt and remove lysosomes, and proteinase inhibitors were included in extraction buffer to prevent proteolysis of PDH complex. Livers (two to eight rats) were disrupted (Potter-Elvehjem homogenizer) in sucrose medium (0.25 M-sucrose/5 mM-Tris/2 mM-EGTA, pH 7.5) (160 mg of tissue/ml). After a low-speed spin (600 g for 10 min) the pellets were re-extracted with a similar volume of sucrose medium. The mitochondrial pellets obtained from the combined supernatants (10000 g for 10 min) were taken up in sucrose medium (4 mg of protein/ml) and incubated for 2 min at 0 °C with digitonin (0.4 mg/ml). The incubation mixture was centrifuged (600 g for 5 min) to remove digitonin, and mitochondria were isolated (10000 g for 10 min), resuspended and incubated (4 mg of protein/ml) for 30 min in KCl medium containing 10 μ M-carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (to effect conversion of inactive PDH complex into active complex). When mitochondria were to be used solely as a source of kinase activator protein, the pellets were taken up in sucrose medium without incubation at 30 °C (80 mg of mitochondrial protein/ml). With either procedure, mitochondrial pellets were then separated (10000 g for 10 min), frozen (liquid N₂), and extracts were prepared by freezing and thawing (three times) in 50 mM-potassium phosphate/10 mM-EGTA/2 mM-dithiothreitol/1 mM-benzamidine/1 mM-phenylmethanesulphonyl fluoride/0.3 mM-tosyl-lysylchloromethane, pH 7.0 (50 mg of protein/ml). In some experiments [Table 1 (lines 1–3) and Table 2] the medium also contained 2% (v/v) ox serum.

Fractionation procedure

Gel filtration of mitochondrial extracts. Samples of extracts of mitochondria incubated in KCl medium/CCCP were retained for assay of PDH kinase activity. After clarification of the extracts by centrifugation at 36000 g for 30 min, clear supernatant fraction (2.5–3 ml) containing approx. 5 units of PDH complex was loaded on to a 140 ml (70 cm \times 1.6 cm) column of Sephacryl S-300 equilibrated with mitochondrial extraction buffer (no ox serum) and eluted with the same buffer (0.25 ml/min;

1 ml fractions). PDH complex (located by assay) was eluted at 38–46 ml, and the three peak tubes were pooled (recovery > 50%; approx. 0.5 unit/ml). Kinase activator protein (located by assay) was eluted at 58–75 ml, and all fractions were pooled.

Gel filtration of purified pig heart PDH complex.

Purified pig heart complex (120 units, 3 ml) in 20 mM-potassium phosphate/2 mM-dithiothreitol was loaded [with addition of glycerol to 2% (v/v), to increase specific gravity] and eluted with mitochondrial extraction buffer as above (recovery > 80%). If necessary, the pooled fractions were concentrated with Centriflo cones.

Rat liver kinase activator protein. Extracts of unincubated mitochondria (20–40 ml) were centrifuged at 150000 g for 2 h to precipitate PDH complexes. Kinase activator protein was then precipitated from the supernatant by addition of (NH₄)₂SO₄ to 243 mg/ml. After stirring for 10 min at 0 °C, the precipitate was collected (36000 g for 15 min) and taken up in 1–2 ml of extraction buffer. The preparation was freed of (NH₄)₂SO₄ either by dialysis for 18 h at 2 °C against 2 litres of extraction buffer, or by chromatography on a 9 cm \times 2.7 cm (50 ml) column of Sephadex G-25 (medium grade) (1 ml fractions were collected, at 2 ml/min; kinase activator protein was eluted at 17–26 ml, cf. Blue Dextran at 18–22 ml).

Rat liver PDH complex. In some experiments PDH complex was partially purified from mitochondria incubated in KCl medium/CCCP after extraction with buffer (see above) containing 2.5% (v/v) Triton X-100. Branched-chain 2-oxoacid dehydrogenase complex was removed by precipitation with 2% (v/v) poly(ethylene glycol) at pH 7.5 in the presence of MgCl₂ added to 10 mM. The supernatant was adjusted to pH 6.5, with 10% (v/v) acetic acid; PDH complex was precipitated by adding poly(ethylene glycol) to 10% (v/v), and after centrifugation was taken up in mitochondrial extraction buffer. The yield was approx. 65% and the activity of branched-chain complex was 1–3% of that of PDH complex. Complete removal of branched-chain complex was effected by precipitation at pH 6.25 (10% acetic acid), and PDH complex was then precipitated and separated from proteins of lower *M_r* at pH 7 by centrifugation at 150000 g for 90 min. The overall yield was approx. 25%. Attempts to effect complete purification by the method used for pig heart PDH complex (Kerbey *et al.*, 1979) were unsuccessful, mainly because the procedure failed to effect separation of rat liver PDH complex from 2-oxoglutarate dehydrogenase complex.

Assays

Protein was assayed by the method of Gornall *et al.* (1949) or with Coomassie Blue (Bradford, 1976) with bovine serum albumin standards, PDH complex by coupling to arylamine acetyltransferase as described by Cooper *et al.* (1974), except that lipoamide dehydrogenase was added to 20 units/ml, and branched-chain complex by the method of Patston *et al.* (1984).

PDH kinase was assayed routinely by the rate of ATP-dependent inactivation of PDH complex at 30 °C in 222 μ l of extraction buffer containing 1 mM-MgCl₂, oligomycin B (34 μ g/ml), lipoamide dehydrogenase (20 units/ml), PDH complex (0.3 unit/ml) and ethanol

(0.8%, v/v; as solvent for oligomycin B). Details of other additions (kinase activator protein) are given in the Results section. After warming to 30 °C (2 min), reaction was initiated by addition of ATP to 0.5 mM, and samples (50 µl) were taken for assay of PDH complex at four time points chosen so that 60–75% inactivation was achieved by the last time point. Control incubations (no ATP) were included routinely. PDH kinase activity is expressed as the apparent first-order rate constant (min^{-1}) calculated by least-squares linear-regression analysis. In some experiments with partially purified rat liver PDH complex or highly purified pig heart PDH complex, PDH kinase was also assayed by the rate of incorporation of ^{32}P from [γ - ^{32}P]ATP into the complex. Incorporation of ^{32}P into protein was measured by the paper-squares method of Corbin & Reimann (1974). Samples were subjected to SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970), and autoradiographs were prepared (Kerbey *et al.*, 1979) to check that incorporation was confined to the α -chain of the E1 component of PDH complex. ATPase activity was assayed with [γ - ^{32}P]ATP as described by Cooper *et al.* (1974). PDH phosphatase activity under the conditions of Denton *et al.* (1972), by using fully phosphorylated pig heart PDH [^{32}P]phosphate complex prepared by the method of Sugden *et al.* (1978). Kinase activator protein in fractions obtained by Sephacryl S-300 chromatography was assayed by the method of Kerbey *et al.* (1984).

One unit of enzyme forms 1 µmol of product/min at 30 °C.

RESULTS AND DISCUSSION

In measurements of PDH kinase activity the following essential criteria were fulfilled. Complete conversion of inactive PDH complex into active complex was achieved before extraction from mitochondria (cf. Fatania *et al.*, 1986). ATPase activities over the full duration of PDH kinase assays were acceptable, being (% of ATP hydrolysed) < 16% for rat liver mitochondrial extracts,

< 4% for rat liver PDH complex + kinase activator protein fraction, and zero for pig heart PDH complex + rat liver kinase activator protein fractions. PDH phosphatase activity was zero under the conditions of PDH kinase assay and, in confirmation of this finding, 50 mM-NaF had no effect on the measured activities of PDH kinase (results not shown). In all experiments there was no loss of PDH complex activity over at least 8 h at 0 °C and 7 min at 30 °C (the longest periods tested). The validity of PDH kinase assays based on ATP-dependent inactivation was assessed by least-squares linear-regression analysis of the first-order plot. In all cases the plots were linear, the correlation coefficient (r) was > 0.93 (usually > 0.99) and the zero-time intercept was close to 100% of the PDH complex activity of the minus-ATP control.

In experiments with rat liver mitochondria, mitochondria were prepared on day 1 and extracts were prepared, gel filtration was accomplished and assays were completed on day 2. In comparing fed and starved, the contents of PDH complex in extracts loaded on Sephacryl S-300 were comparable (fed, 4.3 units; starved, 4.4 units); the elution volumes of fractions 1 (PDH complex) and 2 (kinase activator protein) were comparable for fed and starved, and the recoveries of PDH complex were comparable (55% for fed, 59% for starved).

Effects of starvation (48 h) on the activities of PDH kinase in rat liver mitochondrial extracts and in rat liver PDH complex and on the activity of rat liver kinase activator protein

As shown in Table 1 (line 1) the PDH kinase activity of extracts of rat liver mitochondria was increased 2.2-fold by 48 h starvation of rats. This difference was maintained when extracts were prepared with Triton X-100 (line 4), when PDH complex was precipitated at pH 6.5 with 10% poly(ethylene glycol) (line 5) and further purified by removal of other proteins at pH 6.25, followed by precipitation of PDH complex at 150000 g (line 6). In extracts of rat liver mitochondria gel-filtered on Sephacryl S-300, the PDH kinase activity of PDH

Table 1. Effect of starvation on PDH kinase activities in extracts of rat liver mitochondria or fractions therefrom

PDH kinase activity is expressed as the apparent first-order rate constant for ATP-dependent inactivation of PDH complex (0.3 unit/ml in assay) in extracts of liver mitochondria or fractions therefrom. Results are means \pm S.E.M. for the number of preparations, which was three for lines 1–3, five for line 4, and six for line 5 (each preparation was from four to eight livers, and there were three to eight PDH kinase assays for each preparation). In line 6 mean \pm S.E.M. is for four assays on a single preparation from eight livers. In line 3 the concentration of fraction 2 in the assay was 50% (v/v). For further details see the Experimental section. * $P < 0.01$ for difference from unity; † $P < 0.01$ for difference from fed; ‡ $P < 0.01$ for effect of gel filtration; § $P < 0.01$ for effect of fraction 2. For other differences $P > 0.05$.

Mitochondrial extract or fraction	PDH kinase activity (min^{-1})		
	Fed rats (f)	Starved rats (s)	Ratio (s/f)
1. Unfractionated extract	1.35 \pm 0.08	2.97 \pm 0.19†	2.20 \pm 0.02*
2. Sephacryl S-300, fraction 1 (PDH complex)	0.23 \pm 0.06‡	1.16 \pm 0.19†‡	5.04 \pm 0.36*
3. As, 2 plus corresponding fraction 2 (kinase activator protein)	2.31 \pm 0.24§	5.58 \pm 0.38†	2.47 \pm 0.30*§
4. Unfractionated extract (2.5% Triton X-100)	1.54 \pm 0.11	3.68 \pm 0.10†	2.33 \pm 0.12*
5. PDH complex from 4 [precipitate pH 6.5, 10% poly(ethylene glycol)]	3.01 \pm 0.22	6.44 \pm 0.42†	2.14 \pm 0.22*
6. PDH complex from 5 (150000 g precipitate from pH 6.25 supernatant)	1.40 \pm 0.06	4.01 \pm 0.10†	2.86 \pm 0.12*

Table 2. PDH kinase activities of rat liver mitochondrial PDH complexes of fed or starved (48 h) rats in the absence or in the presence of kinase activator protein from liver mitochondria of fed and starved rats

Extracts of rat liver mitochondria were subjected to gel filtration on Sephacryl S-300 to separate PDH complex (fraction 1) and kinase activator protein (fraction 2). For other details see the legend to Table 1 pertaining to lines 1–3 therein, and the Experimental section. * $P < 0.01$ for difference from unity; † $P < 0.01$ for difference from fed (fraction 1); ‡ $P < 0.01$ for difference from fed (fraction 2). For other differences $P > 0.05$.

Source of fraction 2	PDH kinase activity (min^{-1}) for PDH complex (fraction 1) from:		
	Fed rats (f)	Starved rats (s)	Ratio (s/f)
1. None	0.23 ± 0.06	$1.16 \pm 0.19^\dagger$	$5.04 \pm 0.36^*$
2. Fed rats (F)	2.31 ± 0.24	2.62 ± 0.28	1.15 ± 0.13
3. Starved rats (S)	$5.77 \pm 0.22^\ddagger$	$5.58 \pm 0.38^\ddagger$	0.97 ± 0.05
4. Ratio (S/F)	$2.50 \pm 0.24^*$	$2.13 \pm 0.23^*$	—

complex was decreased by 83% (fed) or 61% (starved) and the ratio (starved/fed) increased to 5 (line 2, cf. line 1). Addition of a second fraction containing kinase activator protein increased PDH kinase activity of PDH complex and restored the ratio (starved/fed) to 2.5 (line 3; cf. lines 2 and 1). The PDH kinase activities in the presence of kinase activator protein were greater (approx. 1.8-fold) than in the original mitochondrial extract. It is suggested that this is due to removal of low- M_r inhibitors of PDH kinase by gel filtration (e.g. thiamin pyrophosphate, ADP, CoA).

The results of cross over experiments are shown in Table 2 (i.e. mixing fractions 1 containing PDH complexes from livers of fed and of starved rats with fractions 2 containing kinase activator protein from fed or starved). When PDH complexes (fraction 1) from fed and starved rats were mixed with fraction 2 from fed rats, the activities of PDH kinase did not differ significantly (line 2). Likewise, when PDH complexes (fraction 1) from fed and starved rats were mixed with fraction 2 from starved rats, the activities of PDH kinase did not differ significantly (line 3) and the activities were 2.1–2.5-fold greater than with fraction 2 from fed rats (line 4). These data suggested that it is the activity of kinase activator protein which is responsible for the increased PDH kinase activity in extracts of liver mitochondria induced by starvation of rats. The data suggested further that the increase in PDH kinase activity in fraction 1 (PDH complex separated by Sephacryl S-300 chromatography) induced by starvation or in PDH complexes separated by poly(ethylene glycol) fractionation is due to tight binding of kinase activator protein to PDH complex. It was not possible to confirm this conclusion by further gel filtration, because of dilution and losses occasioned by the first gel filtration. Attempts to concentrate PDH complex were unsuccessful because of loss of activity.

It was important to confirm these differences in PDH kinase activity by measurements of [^{32}P]phosphorylation of rat liver PDH complex. This was not possible with rat liver mitochondrial extracts or Sephacryl eluates, because of interference by other [^{32}P]phosphorylations (results not shown). PDH complexes purified by poly(ethylene glycol) fractionation and incubated with [γ - ^{32}P]ATP showed a single band of ^{32}P on autoradiographs of SDS gels corresponding in M_r to E1 α of PDH complex; the rate of incorporation of ^{32}P was increased

approx. 2-fold in PDH complex from starved rats as compared with fed controls (results not shown).

Experiments with rat liver kinase activator protein and purified pig heart PDH complex

These experiments were designed primarily to study effects of kinase activator protein from fed and starved rats on a single preparation of PDH complex and the effects of repeated gel filtration. Pig heart PDH complex was used because more was available and it could be purified extensively (see the preceding section). The PDH complex used in these studies was gel filtered on Sephacryl S-300 before use because of the possible presence of kinase activator protein. Siess & Wieland (1982) found that extensive dialysis of pig heart PDH complex led to a decrease in PDH kinase activity which was restored by CoA ($K_{0.5} = 1.8 \mu\text{M}$) and it was important to test the effect of CoA on gel-filtered pig heart PDH complex. No increase in PDH kinase activity was observed with $22.5 \mu\text{M}$ -CoA (results not shown).

The activity of PDH kinase in gel-filtered pig heart PDH complex was increased by kinase activator protein from liver mitochondria of fed or starved rats. The concentration–rate-constant (k) relationships are shown in Fig. 1, together with computed values of k_{max} and μ of kinase activator protein preparation giving $0.5 V_{\text{max}}$. At k_{max} , kinase activator protein from fed rats gave a 35-fold increase in rate constant, and that from starved rats gave an 82-fold increase. The ratio (starved k_{max} /fed k_{max}) was 2.38 ± 0.26 ($P < 0.001$ for difference from unity). There was no significant difference between fed and starved rats in respect of μ of kinase activator protein preparation giving $0.5 k_{\text{max}}$. This may indicate that the preparative procedure gave equivalent recoveries of kinase activator protein from liver mitochondria of fed and starved rats. This is important because interpretation of the results in Table 1 (line 3), Table 2 (line 4) and Table 3 (lines 3 and 4 and lines 10 and 11) assumes equivalent recoveries. The effect of kinase activator protein (50%, v/v in PDH kinase assays) was demonstrable at a higher concentration of PDH complex (2.5 units/ml). The rate constants (min^{-1} ; means \pm S.E.M. for three experiments) were: PDH complex alone 0.23 ± 0.01 ; with kinase activator protein 0.85 ± 0.03 (fed) and 1.67 ± 0.09 (starved). Data in Fig. 2 show that kinase activator protein stimulates [^{32}P]phosphorylation of pig heart PDH complex (starved > fed), and the

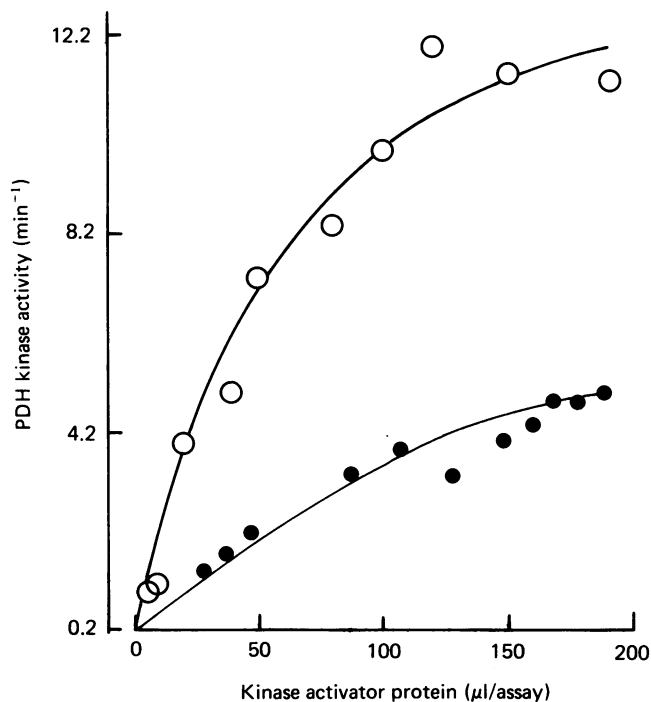


Fig. 1. Concentration-rate relationships for activation of pig heart PDH kinase by kinase activator protein from liver mitochondria of fed (●) or starved (○) (48 h) rats

Rates are expressed as the apparent first-order rate constant for ATP-dependent inactivation of highly purified pig heart PDH complex subjected to gel filtration on Sephacryl S-300. Kinase activator protein was prepared from 150000 g supernatant fractions of extracts of mitochondria from four rat livers by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration on Sephadex G-25. Protein concentrations (fed and starved) were 3.75 mg/ml. Incubation concentrations (PDH kinase assays) were: PDH complex, 0.3 unit/ml; kinase activator protein, volume as shown, in 222 μl final volume. For further details see the Experimental section. Samples were taken for assay of PDH complex at four time points which depended on the rate of inactivation. There were two or three assays at each concentration. Computed values for k_{max} (mean \pm S.E.M., min^{-1}) were 7.27 ± 0.70 (fed) and 17.3 ± 1.83 (starved), ratio (starved/fed) 2.38 ± 0.26 [$P < 0.001$ for (starved - fed) and difference from unity respectively]; and computed values for μl of kinase activator protein/222 μl giving $0.5 V_{\text{max}}$ (mean \pm S.E.M.) were 104 ± 19 (fed) and 81 ± 18 (starved) [$P > 0.3$ for (fed - starved)] (method of Wilkinson, 1961). The rate constant for PDH complex alone was 0.21.

autoradiographs of SDS/polyacrylamide-gel tracks after 5 min of incubation showed only one band of ^{32}P (Fig. 3) corresponding to $\text{E1}\alpha$ of PDH complex (results not shown). The incorporation of ^{32}P in nmol/unit of complex inactivated was as expected from previous studies (cf. legend to Fig. 2 and Kerbey *et al.*, 1979).

As shown in Table 3, the effect of kinase activator protein from starved rats on PDH kinase activity of pig heart PDH complex persisted after gel filtration on Sephacryl S-300 (cf. Table 3, lines 2, 4, 6). The much smaller effect of kinase activator protein from fed rats was no longer statistically significant after gel filtration (cf. Table 3, lines 2, 3, 5). A further gel filtration on

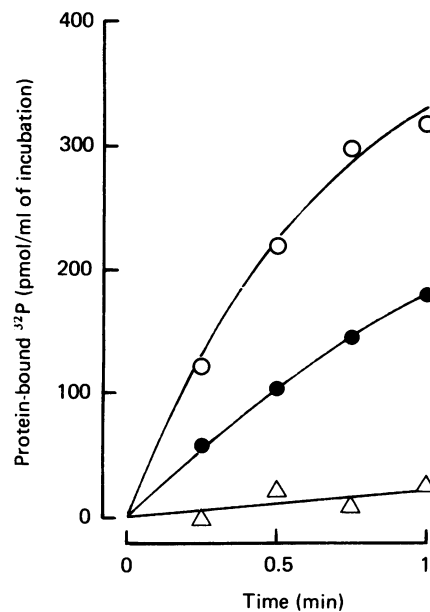


Fig. 2. ^{32}P Phosphorylation of purified pig heart PDH complex in the absence (Δ) or presence of kinase activator protein from liver mitochondria of fed (●) or starved (○) (48 h) rats

Details of the preparations of pig heart PDH complex and of kinase activator protein were as in the legend to Fig. 1. Concentrations were: PDH complex, 0.3 unit/ml; kinase activator protein, 0 or 65 $\mu\text{l}/\text{ml}$ of incubation (fed or starved); $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 0.5 mM (180 d.p.m./pmol). Samples (40 μl) were taken for assays of protein-bound ^{32}P at times shown. For further details see the Experimental section. Each point is the mean of four incubations. At each time point $P < 0.01$ for effect of kinase activator protein; at 0.5, 0.75 and 1 min $P < 0.001$ for difference (fed - starved); at 0.25 min $P < 0.05$ for difference (fed - starved). Incorporations of ^{32}P (nmol of P/unit of PDH complex inactivated at 0.25, 0.5, 0.75 and 1 min) were 0.68, 0.69, 0.77, 0.70 (starved) and 0.53, 0.53, 0.58 and 0.60 (fed). Apparent first-order rate constants for ATP-dependent inactivation (means \pm S.E.M.; min^{-1}) were 0.62 ± 0.03 (fed) and 1.16 ± 0.10 (starved) [$P < 0.01$ for difference (starved - fed)].

Sephacryl S-300 removed the effect of kinase activator protein from starved rats (cf. Table 3, lines 2, 6, 8). These results may indicate that kinase activator protein from starved rats binds to PDH complex and is retained on gel filtration, being separated on a further gel filtration. It was not possible to detect kinase activator protein in later fractions from the second gel filtration, presumably because the concentrations were too low. The results of SDS/polyacrylamide-gel electrophoresis (Coomassie Blue staining) are shown in Fig. 4. PDH complex showed five major bands, corresponding to E2, E3, band 5 [Kerbey & Randle (1985); equivalent to band X of Rahmatullah & Roche (1985) and De Marcucci *et al.* (1985)], $\text{E1}\alpha$, and $\text{E1}\beta$ (Fig. 4, tracks 1, 4). After gel filtration of an admixture with kinase activator protein from fed or starved rats, pig heart PDH complex showed two further bands, of M_r 95000 and 48700 (shown for starved rats in Fig. 4, track 2). These additional bands disappeared after a further gel filtration (Fig. 4, tracks 6-9). These conclusions were confirmed by reflectance

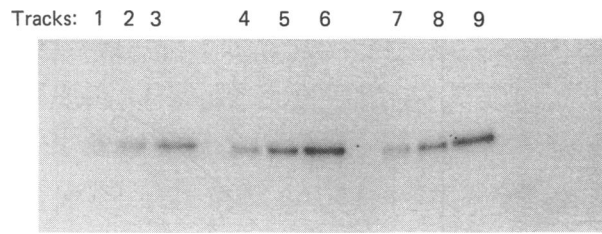


Fig. 3. Autoradiograph of SDS/polyacrylamide-gel electrophoresis of pig heart PDH complex incubated for 5 min (1 unit/ml) with [γ - 32 P]ATP (0.5 mM; 180 d.p.m./pmol) in the absence (tracks 1–3) or in the presence of kinase activator protein (220 μ l/ml of incubation) from fed (tracks 4–6) or starved (tracks 7–9) rats

Within each group the tracks (left to right) correspond to 4, 8 and 16 μ g of protein.

scans of tracks 1, 2 and 9 in Fig. 4 (i.e. the additional bands in track 2 were not detected in tracks 1 and 9); this showed additionally that band 5 (band X) may have increased approx. 2-fold in track 2 relative to tracks 1 and 9 (results not shown). From a Sephadex G-150 column calibrated with M_r markers of 158 000, 40 400 and 17 000, kinase activator protein was eluted over the range of $M_r = 98\,500$ – $48\,200$ (peak approx. 75 000; results not shown).

Gel filtration of pig heart PDH complex on Sephacryl S-300 had no obvious effect on the activity of PDH kinase (Table 3, lines 1, 2). In a single experiment with PDH complex not subjected to gel filtration, the effect on PDH kinase activity of kinase activator protein from fed rats was absent, and that of kinase activator protein from starved rats was attenuated (Table 3, lines 9–11, cf. lines 2–4). After gel filtration of the admixture on Sephacryl S-300, PDH kinase activity fell to below that of the original PDH complex and the difference (starved – fed) was lost (Table 3, lines 12 and 13; cf. lines 9–11). It appeared from this one experiment that gel-filtered pig heart PDH complex was a more sensitive reagent for the assay of PDH kinase activator protein than unfiltered complex, and it was decided on an empirical basis to use only gel-filtered complex. With such preparations the results, without exception, have been completely reproducible.

General discussion and conclusions

The objectives of the present study were to ascertain whether PDH kinase activator protein is present in liver mitochondria, whether its activity is increased by starvation, whether its activity is such that it may be quantitatively important in the regulation of PDH kinase, whether it may be important to the effect of starvation to enhance phosphorylation of PDH complex *in vivo*, and whether it may be the only mechanism operating in mitochondrial extracts to increase the

Table 3. Effect of starvation on the activity of PDH kinase activator protein; experiments with pig heart PDH complex (PDH kinase)

PDH kinase activity is expressed as the apparent first-order rate constant for ATP-dependent inactivation of pig heart complex (0.3 unit/ml in assay). Results are means \pm s.e.m. for the numbers of observations as follows. In lines 1 and 2, three experiments (i.e. gel filtrations of PDH complex); lines 3–6, six experiments (i.e. six preparations each of kinase activator protein from fed and starved); lines 7 and 8, two experiments (i.e. two preparations each of kinase activator protein from fed and starved). There were three to eight assays of PDH kinase in each experiment and each preparation of kinase activator protein was from one to four livers. Results in lines 9–13 were from a single experiment, and s.e.m. values are based on four PDH kinase assays in each group. In all experiments the concentrations of kinase activator protein from fed and starved rats were equivalent in terms of mg of mitochondrial protein used in the preparation. Two preparations of pig heart PDH complex were used, which gave comparable results. For further details see the Experimental section. ^a $P < 0.01$ for difference from unity; ^b $P < 0.01$ for effect of kinase activator protein; ^c $P < 0.01$ for effect of starvation; ^d $P < 0.01$ for effect of gel filtration; ^e $P < 0.01$ for difference from PDH complex (line 9). For other differences $P > 0.05$.

Source of kinase activator protein (rats)	Pig heart PDH complex	PDH kinase activity of pig heart complex		Effect of kinase activator protein (ratio s/f)
		(min ⁻¹)	(ratio s/f)	
1. None	As made	0.21 \pm 0.03	–	–
2. None	Sephacryl S-300	0.22 \pm 0.05	–	–
3. Fed (f)	As in 2	0.48 \pm 0.08 ^b	2.56 \pm 0.40 ^a	3.9 \pm 1.1 ^a
4. Starved (s)	As in 2	1.23 \pm 0.14 ^{bc}		
5. Fed (f)	From Sephacryl S-300	0.36 \pm 0.06	3.03 \pm 0.42 ^a	5.8 \pm 1.2 ^a
6. Starved (s)	of 3 (line 5) or 4 (line 6)	1.09 \pm 0.07 ^{bc}		
7. Fed (f)	From Sephacryl S-300	0.25 \pm 0.06	1.08 \pm 0.40	1.7 \pm 2.4
8. Starved (s)	of 5 (line 7 or 6) (line 8)	0.27 \pm 0.08 ^d		
9. None	From line 1	0.30 \pm 0.01		
10. Fed (f)	From line 1	0.27 \pm 0.02	1.48 \pm 0.09 ^a	–
11. Starved (s)	From line 1	0.40 \pm 0.003 ^{bc}		
12. Fed (f)	From Sephacryl S-300	0.16 \pm 0.02 ^{de}	1.26 \pm 0.24	–
13. Starved (s)	of 10 (line 12) or 11 (line 13)	0.20 \pm 0.04 ^{de}		

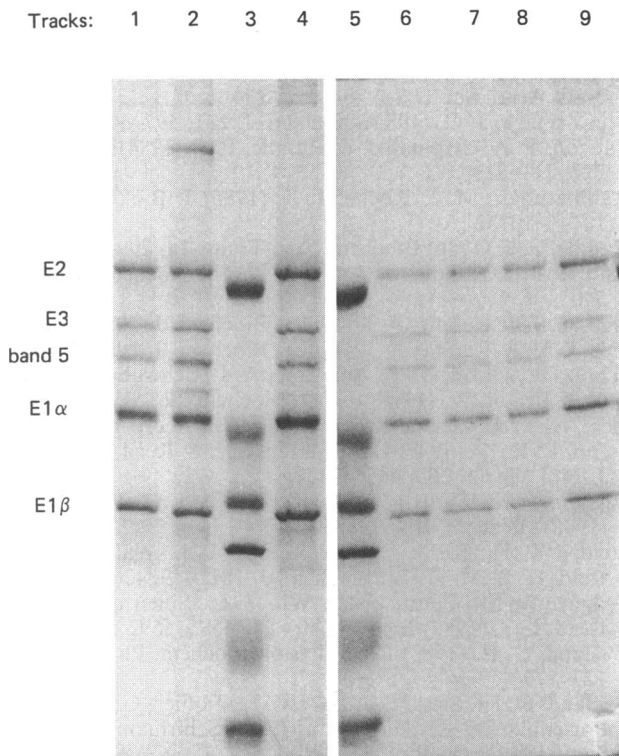


Fig. 4. SDS/polyacrylamide-gel electrophoresis of pig heart PDH complex stained with Coomassie Blue

Tracks 1 and 4, pig heart PDH complex after gel filtration on Sephacryl S-300. Track 2, pig heart PDH complex after gel filtration on Sepacryl S-300 of an admixture with kinase activator protein from starved rats (see Table 3). Tracks 3 and 5, M_r markers ($\times 10^{-3}$): 66, 45, 36, 24, 21.1, 14.2. Tracks 6-9, pig heart PDH complex after a further gel filtration on Sephacryl S-300 of the Sephacryl S-300 fraction shown in track 2. Computed M_r values ($\times 10^{-3}$): tracks 1, 4, 6-9, 67.5 (E2), 57.6 (E3), 52.6 (band 5), 44.9 (E1 α), 34.6 (E1 β); track 2, as tracks 1, 4, 6-9, plus 95 and 48.7.

activity of PDH kinase. The objectives are important, because liver contains a substantial proportion of whole-body PDH complex and it is potentially capable of furnishing sufficient kinase activator protein for purification and characterization.

The present study has shown that rat liver mitochondria contain substantial activity of PDH kinase activator protein and that the activity is increased approx. 2.5-fold by starvation, as in heart and skeletal muscle (cf. Kerbey & Randle, 1982; Fuller & Randle, 1984). The effect of starvation on the activity of kinase activator protein resulted in an increase in the V_{max} of the PDH kinase reaction; the concentration of kinase activator protein required for 0.5 V_{max} was apparently unaffected by starvation. If kinase activator protein is a single molecular species, then this might suggest that starvation results in an increase in specific activity through some stable modification such as covalent modification.

The effects of kinase activator protein on the activity of PDH kinase in pig heart PDH complex were substantial. Under V_{max} conditions an 82-fold increase in PDH kinase activity was computed for preparations

from starved rats and a 35-fold increase for preparations from fed rats. These effects are considerably more marked than those of other activators of PDH kinase (acetyl-CoA, NADH), which are no more than 3-fold (Kerbey *et al.*, 1979). The computed times for 50% inactivation ($t_{0.5}$) of pig heart PDH complex by phosphorylation are illuminating. With kinase activator protein in the present study, computed $t_{0.5}$ was 2.5 s (starved, at k_{max}), 5.7 s (fed, at k_{max}), 3.6 s (starved, at highest experimental concentration used) and 8.7 s (fed, at highest experimental concentrations used); $t_{0.5}$ for gel-filtered PDH complex alone was 3.5 min. In our experience (A. L. K. & P. J. R.) of some 100 preparations of PDH complex phosphorylated at 2.5-30 units/ml the lowest $t_{0.5}$ was 30 s, and generally $t_{0.5}$ was in the range 1.5-2.5 min. It seems possible that the variation in PDH kinase activity between preparations of PDH complex may involve variation in the activity and/or content of bound kinase activator protein.

The cross-over experiments shown in Table 2 have indicated that the difference in PDH kinase activity between mitochondrial extracts from livers of starved and fed rats is conferred mainly and perhaps solely by the activity of kinase activator protein. The experiments do not fully exclude the possibility of a difference in the activity of PDH kinase intrinsic to the complex. However, the gel-filtration experiments with pig heart complex may indicate that apparent differences in intrinsic PDH kinase activity of rat liver PDH complexes are due to the activity of kinase activator protein (starved > fed) retained by the complex after a single gel filtration. This increased PDH kinase activity is associated with the appearance in the pig heart PDH complex fraction of bands on SDS gels of M_r 95000 and 48700. These values approximate to those of the α and β subunits of PDH phosphatase (98000 and 50000; Teague *et al.*, 1982). If these bands are subunits of PDH phosphatase, then they are bound to PDH complex by a Ca^{2+} -independent mechanism, as all operations were carried out in the presence of 10 mM-EGTA and in the absence of added Ca^{2+} . It is unlikely that kinase activator protein has the M_r of PDH phosphatase (140000-150000), as its computed M_r by gel filtration (peak tube) was approx. 75000 (the present study; see also Kerbey *et al.*, 1984).

The effect of starvation to decrease the percentage of active complex in liver (up to 3-fold) is less marked than in muscles (e.g. see Wieland *et al.*, 1973; Wieland, 1983; Randle, 1986; Fatania *et al.*, 1986), whereas the increase in the activity of kinase activator protein is comparable (cf. the present study and Hutson & Randle, 1978; Kerbey & Randle, 1981, 1982). The reason for the smaller decrease in percentage of active complex in liver is not known, but two possibilities are foreseen. Mitochondrial $[Ca^{2+}]$ may be increased in liver in starvation by actions of glucagon and adrenaline (McCormack, 1985), thereby attenuating the decrease in percentage of active complex. Moreover, counter-transport of intramitochondrial acetoacetate and cytosolic pyruvate on the monocarboxylate transporter may increase mitochondrial [pyruvate] and lead to inhibition of PDH kinase (Scholz *et al.*, 1978; Dennis *et al.*, 1978; Zwiebel *et al.*, 1982; Agius & Alberti, 1985). This latter mechanism is unlikely to operate in muscles.

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