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Pyruvate dehydrogenase kinase/activator in rat heart mitochondria

Assay, effect of starvation, and effect of protein-synthesis inhibitors in starvation

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Purified pig heart pyruvate dehydrogenase complex is denuded of its intrinsic pyruvate dehydrogenase kinase activity by sedimentation from dilute solution (60 munits/ml). Kinase activity is restored by a supernatant fraction prepared by high-speed centrifugation of rat heart mitochondrial extracts; the factor responsible is referred to as kinase/activator. Kinase/activator was also assayed by its ability to accelerate MgATP-induced inactivation in dilute solutions of unprocessed complex (50 munits/ml). With this assay it has been shown that the activity of kinase/activator in heart mitochondria is increased 3–6-fold by starvation of rats for 48 h. This increase was prevented completely by cycloheximide treatment and prevented partially by puromycin treatment of rats during starvation. The concentration of kinase/activator in heart mitochondria fell during 20 h of re-feeding of 48 h-starved rats; this fall was correlated with an increase in the proportion of complex in the active form. Kinase/activator was also extracted from ox kidney mitochondria, and on gel filtration (Sephadex G-100, superfine grade) was eluted close to the void volume. Kinase/activator (ox kidney or rat heart) was thermolabile, non-diffusable on dialysis, and inactivated by trypsin. The results of this study appear to show increased cytoplasmic synthesis in starvation of pyruvate dehydrogenase kinase and/or of an activator of the kinase.

After purification, the pyruvate hydrogenase complex (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3) of animal tissues is phosphorylated (MgATP) and inactivated by pyruvate dehydrogenase kinase (EC 2.7.1.99) intrinsic to the complex; re-activation and dephosphorylation is catalysed by a separate mitochondrial phosphatase (EC 3.1.3.43) (Linn *et al.*, 1969*a*,*b*). Starvation or alloxan-diabetes in the rat leads to increased conversion of active (dephosphorylated) pyruvate dehydrogenase complex into inactive (phosphorylated) complex in a number of tissues, including heart muscle (Wieland *et al.*, 1971; Kerbey *et al.*, 1976; Sale & Randle, 1981). Evidence reviewed elsewhere indicates that an increase in the rate of the kinase reaction is a major factor leading to greater phosphorylation and inactivation of the complex in starvation and in diabetes (Randle *et al.*, 1981).

The kinase reaction is accelerated by increased mitochondrial concentration ratios of ATP/ADP, NADH/NAD^+ and acetyl-CoA/CoA. Evidence given by Kerbey *et al.* (1977), Hutson & Randle (1978) and Baxter & Coore (1978) showed that additional factor(s) other than metabolite concentration ratios accelerate(s) the kinase reaction in starved or alloxan-diabetic rats (referred to as 'factor'). The factor was stable because it persisted through isolation, incubation and extraction of rat heart mitochondria (Hutson & Randle, 1978). More recent studies have shown that a factor which accelerates the pyruvate dehydrogenase kinase reaction in extracts of rat heart mitochondria can be separated from the pyruvate dehydrogenase complex by high-speed centrifugation. The factor was non-diffusable on dialysis and thermolabile, and its activity in heart mitochondria was increased by starvation or induction of alloxan-diabetes in the rat (Kerbey & Randle, 1981). The factor could either be free pyruvate dehydrogenase kinase (i.e. not bound to complex) and/or an activator of the kinase (Kerbey & Randle, 1981); it is referred to here as kinase/activator.

In the present study we describe an assay for kinase/activator which utilizes pig heart pyruvate dehydrogenase complex. Conditions have been defined in which pig heart complex is either denuded of its intrinsic kinase or in which the activity of the intrinsic kinase is not expressed. It is shown that the kinase/activator is destroyed by trypsin, that it is increased in activity in heart mitochondria by

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starvation, that the increase in activity induced by starvation is blocked by treatment of the rat with cycloheximide or puromycin, and that it is present in mitochondria from a bulk source (ox kidney).

Experimental

Materials

Cycloheximide, puromycin dihydrochloride and trypsin inhibitor (soya bean) were from Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, U.K. Trypsin [treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK')] was from Millipore Corp, Freehold, NJ, U.S.A. Pyruvate dehydrogenase complex was purified from pig hearts (Kerbey *et al.*, 1979). Dialysis tubing (Visking 8/32) was from the Scientific Instrument Centre Ltd., London W.C.1, U.K.

Methods

Rats. Sources of rats and details of feeding were as given by Kerbey *et al.* (1977). Details of treatments with cycloheximide were as follows. Cycloheximide was given to fed or starved rats by intraperitoneal injection of a solution (1mg/ml) in 0.9% (w/v) NaCl at 11:00h (1mg/kg), 17:00h (2mg/kg) (day 1) and 09:00h (1mg/kg) (day 2). Puromycin was given by intraperitoneal injection of a solution (35mg/ml) in 0.9% NaCl (50mg/kg) at 11:00, 17:00 and 22:00h (day 1) and 09:00h (day 2). Food was withdrawn from starved rats at 11:00h on day 1. Control rats (fed or starved) received 0.9% NaCl. Rats were killed between 11:00 and 14:00h on day 2.

Hearts and heart mitochondria. Rats were anaesthetized with intraperitoneally injected pentobarbitone sodium (Sagatal) (60mg/kg) before removal of the heart. Hearts destined for assay of pyruvate dehydrogenase complex were frozen with a tissue clamp at liquid-N_2 temperature. Extracts were prepared and assayed for active complex and total complex (sum of active and inactive forms) as described by Sale & Randle (1981). Heart mitochondria (unfrozen tissue) were prepared as described by Hutson & Randle (1978); samples (1–1.5mg of protein) were added to 0.5ml of KCl medium, pelleted by centrifugation and aspiration of the supernatant and frozen and stored in liquid N_2 (Kerbey *et al.*, 1976). Extracts (4mg of mitochondrial protein/ml of extraction buffer) and high-speed-supernatant fraction were prepared as described by Kerbey & Randle (1981). The extraction buffer was 30mM-potassium phosphate/10mM-EGTA/1mM-7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK')/10mM-dithiothreitol, pH7.0, containing 25μg of oligomycin/ml.

Assays. Pyruvate dehydrogenase complex (active form) was assayed spectrophotometrically by coupling to arylamine acetyltransferase (Coore *et al.*, 1971). One unit of enzyme complex forms 1μmol of acetyl-CoA/min at 30°C.

Pyruvate dehydrogenase kinase activity was assayed by monitoring the rate of ATP-dependent disappearance of active pyruvate dehydrogenase complex. Routinely the incubation (500μl) contained pig heart complex (50munits/ml), $MgCl_2$ (1mM), ATP (0.5mM) and either mitochondrial extraction buffer or high-speed supernatant fraction (425μl). Control incubations were made under each experimental condition in the absence of ATP. In some experiments (indicated in the text or Figures), pig heart complex was denuded of kinase activity before use by centrifugation at a concentration of 60munits/ml for 90min at 150000**g** and the pellets were dissolved (1 unit/ml) in 20mM-potassium phosphate/2mM-dithiothreitol (pH7.0). Incubation mixtures (without ATP) were warmed to 30°C and the kinase reaction was initiated by addition of ATP to 0.5mM (controls received an equivalent volume of water). Samples (100μl) were taken at not less than four time intervals in the course of incubation for assay of active complex. The loss of activity of complex incubated in the absence of ATP under any condition was <2.5%/min. The high-speed-supernatant fractions of mitochondrial extracts were devoid of pyruvate dehydrogenase complex activity. Pyruvate dehydrogenase phosphate phosphatase in the high-speed supernatant fraction does not reactivate phosphorylated complex under the incubation conditions used (Hutson & Randle, 1978). ATPase activity removed <1.5% of ATP/min; ATPase activity in mitochondrial extracts shows no significant difference between the experimental groups (Hutson & Randle, 1978).

Procedure and calculation of results. Frozen pellets of heart mitochondria were prepared on day 1 and stored in liquid N_2; high-speed-supernatant fraction was prepared and pyruvate dehydrogenase kinase assays were performed on day 2. Kinase/activator in frozen mitochondria was stable for at least 48h at liquid N_2 temperature (results not shown). Different experimental groups and appropriate controls were compared in assays conducted the same day.

Inactivation by ATP in kinase assays was calculated as 100× (activity with ATP)/(activity without ATP). Inactivation by ATP is a pseudo-first-order reaction (Kerbey & Randle, 1981), and the rate of inactivation was expressed as the apparent first-order rate constant calculated ($\pm$S.E.M.) from least-squares linear regression analysis of ln(inactivation by ATP) against time of incubation; this calculation gives negative rate constants. The correlation coefficients (r) were >-0.80 (mean $\pm$ S.E.M. for 49 such analyses was -0.96 ± 0.01); deviations from linearity were not

significant. The intercept on the ordinate, which represents ln (% of active complex at zero time) was 4.68 ± 0.02 (mean ± S.E.M. for 49 observations). This is equivalent to $107.7 \pm 1\%$, which is significantly greater than 100%. The reason for this minor departure from first-order rate kinetics is not clear.

Results and discussion

Assay of rat heart mitochondrial kinase/activator with purified pig heart pyruvate dehydrogenase complex

In first demonstrating kinase/activator in a high-speed-supernatant fraction of mitochondrial extracts, the assay system used was active complex in mitochondrial extracts from hearts of fed normal rats (Kerbey & Randle, 1981). Kinase activity was monitored by ATP-dependent disappearance of active complex or by incorporation of ^{32}P from [γ-^{32}P]ATP; the two measurements gave equivalent results. This method is inconvenient for purifying and characterizing kinase/activator; repeated preparation of mitochondria is time-consuming, and mitochondrial extracts of fed rats contain kinase/activator.

In 1973, in the course of a study of regulation of the kinase reaction in purified pig heart complex it was observed that inactivation of the complex by ATP was essentially absent in dilute solutions of complex (5–10 munits/ml) (R. H. Cooper, A. L. Kerbey & P. J. Randle, unpublished work). This suggested that kinase intrinsic to the complex or alternatively a kinase activator dissociates from the complex at high dilution. It suggested also the possibility that complex may be denuded of kinase (or activator) by sedimentation from dilute solutions at 150000 *g*. It is difficult to distinguish between the alternative possibilities (kinase or kinase activator). The protein assigned as kinase (two subunits, M_r 45000 and 48000; Reed & Pettit, 1981) is not seen on sodium dodecyl sulphate/polyacrylamide-gel

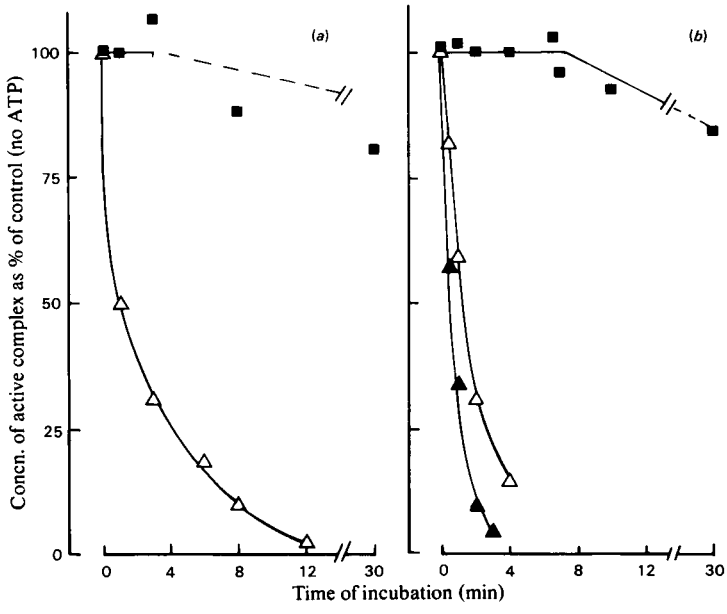

Fig. 1. *Assay of rat heart mitochondrial kinase/activator with purified pig heart pyruvate dehydrogenase complex denuded of kinase activity by sedimentation from dilute solution (60 munits/ml)*

For details of preparation of mitochondrial high-speed-supernatant fraction and denuded complex and enzyme assay see the Experimental section. In panel (*a*) denuded complex (■, 0.37 unit/ml) and unprocessed complex (△, 0.48 unit/ml) were incubated at 30°C with and without 0.5 mM-ATP, and samples were taken for assay of complex (active form) at the times shown. Apparent first-order rate constants for inactivation by ATP were: (■) -0.01 ± 0.005 and (△) -0.3 ± 0.02 (means ± S.E.M.; min^{-1}; for difference $P < 0.001$). In panel (*b*) denuded complex (50 munits/ml) was incubated as in panel (*a*) in the absence (■) or presence of heart mitochondrial high-speed-supernatant fraction from fed rats (△) or 48 h-starved rats (▲) (concentration of fraction in incubation was 85%, v/v). Apparent first-order rate constants for inactivation by ATP were: (■) -0.01 ± 0.005 (15 observations), (△) -0.38 ± 0.04 (19 observations) and (▲) -1.05 ± 0.04 (12 observations) ($P < 0.001$ for effect of supernatant fractions and for difference between fed and 48 h-starved).

electrophoresis of the holocomplex, perhaps because its concentration in the complex is too low.

Two alternative assays for kinase/activator using purified pig heart pyruvate dehydrogenase complex have been explored. The first of these utilizes purified complex sedimented at 150000*g* from a dilute solution (60munits/ml). As shown in Fig. 1.(*a*), complex prepared in this way and incubated at 0.37 unit/ml with ATP showed no inactivation in 3min and only 19% inactivation in 30min. Unprocessed complex at 0.48 unit/ml showed 69% inactivation in 3min and 98% inactivation in 12min (the rate of inactivation of unprocessed complex is the same at 0.3 and 0.5 unit/ml; results not shown). It is well established that complex sedimented repeatedly at high concentration is rapidly inactivated by ATP; this forms part of the procedure for purification of the complex (see, e.g., Kerbey *et al.*, 1979). These results show that sedimentation of the complex in dilute solution results in loss of kinase activity, presumably as a result of dissociation and removal of kinase and/or an associated activator. At 50munits/ml, sedimented complex showed no inactivation by ATP in 7min and only 16% inactivation in 30min (Fig. 1*b*). Rapid inactivation by ATP was restored by high-speed supernatants prepared from extracts of rat heart mitochondria and devoid of pyruvate dehydrogenase complex activity (typical results are shown in Fig. 1*b*). Apparent first-order rate constants with supernatants (min^{-1}; means ± S.E.M.) were -0.38 ± 0.04 (fed rats; 19 observations) and -1.05 ± 0.04 (48h-starved rats; 12 observations) ($P < 0.001$ for difference between fed and starved).

Data in Fig. 2 show that processed complex (i.e. sedimented at 60munits/ml at 150000*g*) failed to incorporate ^{32}P from [γ-^{32}P]ATP during 2min of incubation (concentration of complex 50munits/ml). Incorporation was induced by high-speed-supernatant fraction from mitochondria of fed rats (6.98 ± 0.30pmol/min per ml of incubation mixture; mean ± S.E.M.) or starved rats (8.96 ± 0.41pmol/min per ml) ($P < 0.001$ for difference from zero and between fed and starved). In a further experiment (results not shown in Fig. 2), processed complex (0.5 unit/ml) was incubated with [γ-^{32}P]ATP (219d.p.m./pmol). This particular preparation showed some inactivation by ATP; the concentrations of active complex, as percentages of control without ATP, were 94% (1min), 75% (2min), 68% (3min) and 67% (4min) (means of duplicates). Incorporations of ^{32}P (pmol/ml, means ± S.E.M. for four observations) were 70 ± 6 (2min) and 121 ± 14 (4min). In the presence of high-speed-supernatant fraction (69%, v/v) from starved rats, inactivation by ATP and incorporation of ^{32}P were increased. The concentrations of active complex, as percentages of control without ATP, were 70% (1min), 39% (2min), 23% (3min) and 12% (4min) (means of duplicates); ^{32}P incorporations (pmol/ml; means ± S.E.M. for four observations) were 271 ± 6.8 (2min) and 389 ± 10.8 (4min) ($P < 0.001$ for effect of supernatant fraction at 2 and 4min). These results show that supernatant fraction stimulated phosphorylation of processed pig heart complex and that starvation for 48h increases the activity of the supernatant fraction in this respect.

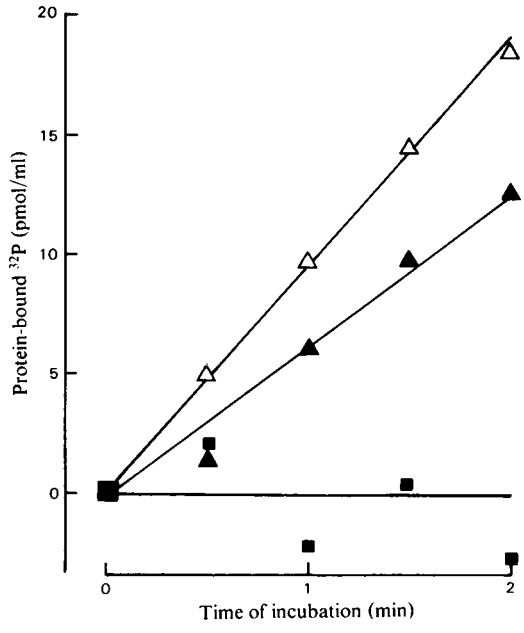

Fig. 2. *Effect of rat heart mitochondrial kinase/activator on incorporation of ^{32}P from [γ-^{32}P]ATP into complex denuded of pyruvate dehydrogenase kinase activity*
Conditions of incubation were as in Fig. 1 (*b*) except for inclusion of [γ-^{32}P]ATP (533d.p.m./pmol). Samples were taken for assay of protein-bound ^{32}P at the times shown and incorporations were corrected for blank values (i.e. ^{32}P incorporation measured in the absence of complex). Incubations were made in the absence (■) or in the presence of heart mitochondrial supernatant fraction from fed (▲) or 48h-starved (△) rats. Rates of incorporation (pmol/min per ml of incubation) were (■) -1.3 ± 1.1 ($P > 0.05$ for difference from zero), (▲) 6.98 ± 0.30 and (△) 8.96 ± 0.41 (means ± S.E.M.; $P < 0.001$ for effect of supernatant fraction and for effect of starvation).

Assays based on incorporation of ^{32}P were less precise than those based on inactivation by ATP, especially at a low concentration of complex (50munits/ml). This is because of corrections applied for blank incorporation (i.e. incorporation in the absence of complex); the blank incorporation was increased by high-speed-supernatant fractions.

At 50munits/ml (Fig. 2) the blank incorporation ranged from approx. 10% of total incorporation at 2min to 50% at 0.5min. At 0.5 unit/ml (see above) the blank incorporations (as percentages of total incorporation with complex present) were 24% (2min) and 20% (4min) in the absence of supernatant fraction and 11% (2min) and 9% (4min) in the presence of supernatant fraction. At low concentrations of complex the assay based on ATP inactivation was more precise, because inactivation of the complex in the absence of ATP is small (<2.5%/min). Because of the greater precision, all subsequent studies were based on inactivation by ATP.

For routine use an assay for kinase/activator was developed which uses unprocessed complex (this is more convenient). In pilot experiments the rate of inactivation of complex by ATP and the effect of mitochondrial high-speed supernatants were investigated at seven concentrations of complex between 40munits/ml and 4 units/ml. Results at two of these concentrations are shown in Fig. 3. At 0.5 unit/ml (panel *a*) and at 0.3 unit/ml (results not shown), complex was completely inactivated with ATP alone after 3min. Addition of mitochondrial high-speed supernatant from fed rats had no effect on the rate of inactivation, whereas some acceleration was seen with high-speed supernatant from 48h-starved rats. At 40munits/ml (panel *b*) and at 60, 80 and 110units/ml (results not shown), there was no inactivation of complex in 3min by ATP alone. Inactivation by ATP was induced by high-speed supernatants from fed or 48h-starved rats. At 40munits/ml the apparent first-order rate constants with supernatants (means ± S.E.M.; min^{-1}) were -0.79 ± 0.04 (fed rats) and -1.82 ± 0.08 (48h-starved) ($P < 0.001$ for difference between fed and starved). At 2.25 and 4 units/ml, neither high-speed supernatant accelerated ATP-induced inactivation of the complex (results not shown).

These results show that high-speed-supernatant fraction prepared from rat heart mitochondrial extracts increased the rate of inactivation of purified pig heart pyruvate dehydrogenase complex by ATP. High-speed supernatant from heart mitochondria of 48h-starved rats was significantly more active than supernatant from fed rats. The findings of Kerbey & Randle (1981) with an assay based on complex in mitochondrial extracts are thus confirmed with assays based on purified pig heart pyruvate dehydrogenase complex. This assay has been used in the further experiments described below.

Effect of concentration of high-speed-supernatant fraction of heart mitochondrial extracts on rate of inactivation of pig heart pyruvate dehydrogenase complex by ATP

The results given in the preceding section may suggest that the concentration of kinase/activator in heart mitochondria is increased by 48h of starvation in the rat. In an attempt to test the validity of this assumption and to assess the relative concentrations in mitochondria of fed and starved rats, kinase/activator was assayed at several concentrations of high-speed-supernatant fractions. The results are shown in Fig. 4 as a plot of apparent first-order rate constant (K) against concentration of supernatant fraction. This is assumed to be equivalent to a plot of (initial velocity of inactivation by ATP) against (concentration of supernatant fraction). The initial concentration of complex was fixed at 50munits/ml, the concentration of ATP was approx. $20 \times K_m$, and ATP disappearance was <5%.

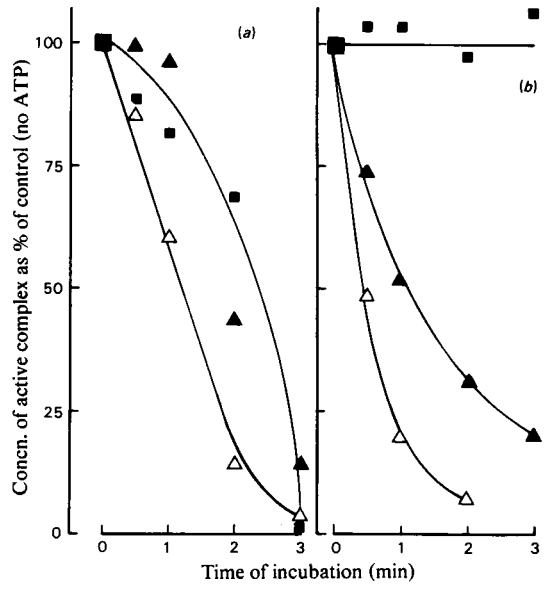

Fig. 3. *Assay of rat heart mitochondrial kinase/activator with unprocessed pig heart pyruvate dehydrogenase complex*

Pig heart pyruvate dehydrogenase complex [0.5 unit/ml in (*a*); 0.04 unit/ml in (*b*)] was incubated with and without ATP (0.5mM) and samples were taken for assay of active complex at the times shown. (■) Incubations without mitochondrial high-speed supernatant fraction; incubations with supernatant fraction (85%, v/v), (▲) from fed rats and (△) from 48h-starved rats. Apparent first-order rate constants for inactivation by ATP (min^{-1}; means ± S.E.M.) were: (*a*), (■) -0.29 ± 0.03 (▲) -0.37 ± 0.10, (△) -0.85 ± 0.10 ($P < 0.001$ for differences from zero and for effect of supernatant fraction from 48h-starved rats); (*b*), (▲) -0.79 ± 0.04, (△) -1.82 ± 0.08 ($P < 0.001$ for effect of both supernatant fractions and for effect of 48h starvation).

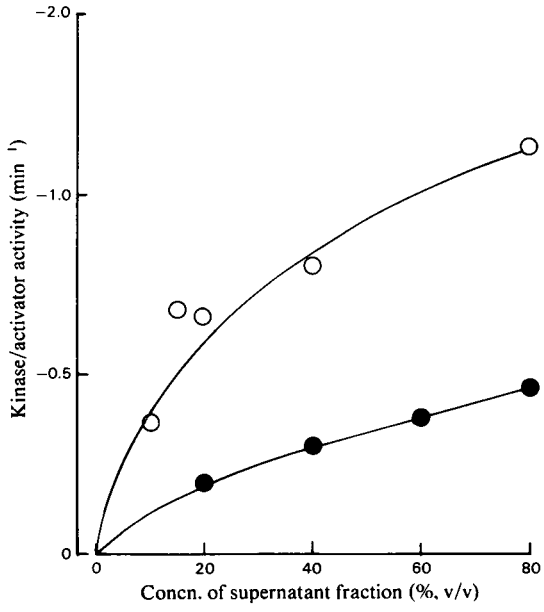

Fig. 4. *Quantitative effects of kinase/activator in heart mitochondrial supernatant fraction assayed with unprocessed pig heart complex*

Unprocessed pig heart pyruvate dehydrogenase complex (50 munits/ml) was incubated in the presence and in the absence of ATP (0.5 mM), and samples were taken for assay of active complex at 0.5, 1.0, 1.5 and 2.0 min. The concentration of heart mitochondrial supernatant fraction was as shown on the abscissa. Apparent first-order rate constants for inactivation by ATP were calculated from the ratio [(active complex in the presence of ATP)/(active complex in the absence of ATP)]. The rate constant in the absence of supernatant fraction ($-0.16 \pm 0.05\ min^{-1}$; mean ± S.E.M. for 15 observations) was subtracted from rate constants obtained with supernatant fractions to give the values shown in the Figure. (●) Supernatant fraction from fed rats; (○) supernatant fraction from 48 h-starved rats.

The data were analysed, on an empirical basis, by a computer program (Jones, 1970) for the Lineweaver–Burk equation, substituting the apparent first-order rate constant (k) for initial velocity, and the concentration of supernatant fraction for substrate concentration. This gave values for $K_{max.}$ (maximum rate constant; min^{-1}; mean ± S.E.M.) of -0.86 ± 0.09 (supernatant fraction, fed rats) and -1.39 ± 0.22 (supernatant fraction, starved rats) ($P > 0.05$ for difference between fed and starved). The concentrations of supernatant fraction giving 0.5 $K_{max.}$ (mean ± S.E.M. for percentage concentration of supernatant fraction) were 73 ± 13 (supernatant fraction, fed rats) and 24 ± 9 (supernatant fraction starved rats) ($P < 0.05$ for difference between fed and starved). This might suggest that the concentration of kinase/activator in supernatant fraction from starved rats is approximately 3 times that in the fed [(ratio of percentage concentrations of supernatant fraction in the assay giving 0.5 $K_{max.}$)$^{-1}$]. No conclusions are drawn from this analysis about the nature of kinase/activator, i.e. whether kinase and/or activator. The data have also been analysed more empirically by least-squares linear-regression analysis of log (concentration of supernatant fraction) against (apparent first-order rate constant). Deviations from linearity were not significant, the correlation coefficients (r) were >0.95, and the slopes did not differ significantly (plots not shown). Relative activities by this method of analysis (starved/fed) were 6.3:1.

Effect of protein-synthesis inhibitors on kinase/activator in heart mitochondria of fed and starved rats

In describing kinase/activator in mitochondrial high-speed supernatant (Kerbey & Randle, 1981), evidence was given that the kinase/activator is thermolabile and non-diffusable on dialysis. This suggested that the kinase/activator is a protein. Further evidence for this is provided by experiments which show that kinase activator is inactivated by trypsin. In these experiments high-speed-supernatant fraction from starved rats was incubated with trypsin (0.1 mg/ml) for 30 min at 30°C and assayed for kinase activator after addition of soya-bean trypsin inhibitor (0.15 mg/ml). Kinase/activator was detected in high-speed-supernatant fraction incubated alone or with trypsin inhibitor, but kinase/activator was not detected in high-speed supernatant incubated with trypsin (results not shown). The combination of (trypsin + trypsin inhibitor) did not inactivate pig heart pyruvate dehydrogenase in the kinase/activator assay.

In view of evidence that kinase/activator is a protein and increased in concentration by starvation, it seemed appropriate to investigate effects of protein-synthesis inhibitors on the increase in activity of kinase/activator induced by starvation. In the present study, cycloheximide and puromycin were used successfully at concentrations reported to induce near-maximal inhibition of protein synthesis (Rothblum *et al.*, 1976; Greengard *et al.*, 1963). In these experiments it was necessary to restrict the period of starvation to 24 h; few rats survived 48 h of treatment with these drugs at the doses employed. The relative activities of kinase activator (as apparent first-order rate constants; min^{-1}; means ± S.E.M.) after 0, 20 and 42 h of starvation were -0.89 ± 0.068, -1.14 ± 0.04 and -1.39 ± 0.035 respectively ($P < 0.01$ for zero versus 20 h, zero

versus 42h and 20h versus 42h). Experiments involving actinomycin treatment were abandoned because of the high mortality during a period of 24h of administration.

The effects of 24h of treatment with cycloheximide on activity of kinase/activator in mitochondrial supernatant fraction of fed or 48h-starved rats is shown in Fig. 5. Assays were with pig heart complex; other details are in the Figure. In the absence of supernatant fraction, no significant inactivation by ATP was detected (apparent first-order rate constant -0.08 ± 0.034; mean ± S.E.M. for five observations). The rate constants for ATP-induced inactivation with high-speed supernatants were -1.22 ± 0.10 (24h starved) and -0.68 ± 0.1 (fed) (means ± S.E.M. for five observations; $P < 0.001$ for difference between starved and fed). Administration of cycloheximide during starvation decreased the rate constant to -0.74 ± 0.08 ($P < 0.001$ for effect of cycloheximide). Administration of cycloheximide to fed rats had no effect; the rate constant was -0.60 ± 0.07. Thus cycloheximide completely prevented the rise in kinase/activator activity induced by starvation while having no significant effect in fed rats.

Qualitatively similar results were obtained in experiments with puromycin. The rate constants (means ± S.E.M.; 10–15 observations) were -0.88 ± 0.03 (starved, control), -0.54 ± 0.04 (fed, control), -0.74 ± 0.04 (starved, +puromycin) and -0.60 ± 0.04 (fed, +puromycin) ($P < 0.001$ for effect of starvation in absence of puromycin; $P < 0.01$ for effect of puromycin in starved; $P < 0.02$ for effect of starvation during puromycin treatment). Thus puromycin partially inhibited (50%) the rise in kinase/activator activity induced by starvation.

These results indicate that starvation may increase the rate of cytoplasmic synthesis of a protein which is destined to be either mitochondrial pyruvate dehydrogenase kinase and/or an activator of the pyruvate dehydrogenase kinase reaction.

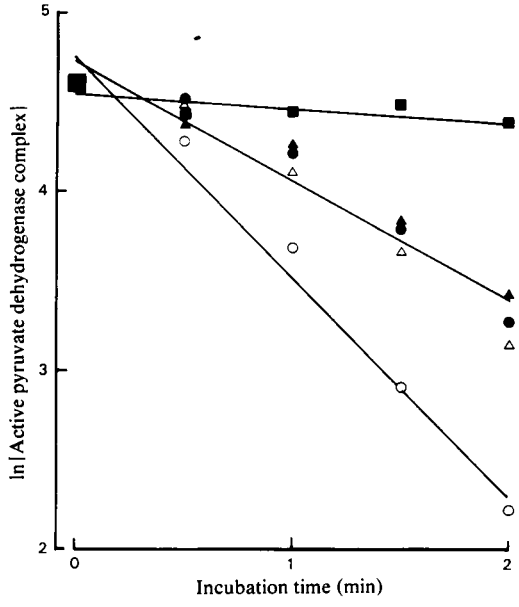

Fig. 5. *Effect of treatment of rats with cycloheximide on the activity of kinase/activator in heart mitochondria of fed and 24h-starved rats*

For details of treatment of rats with cycloheximide and preparation of heart mitochondrial supernatant fractions, see the Experimental section. Kinase/activator was assayed as in Fig. 4, but at a fixed concentration of supernatant fraction (85%, v/v). Incubations were: (■) absence of supernatant fraction, (●) supernatant fraction from fed rats injected with saline, (▲) supernatant fraction from fed rats injected with cycloheximide, (○) supernatant fraction from 24h-starved rats injected with saline, (△) supernatant fraction from 24h-starved rats injected with cycloheximide. Each point is the mean of five observations on two mitochondrial preparations. The apparent first-order rate constants (means ± S.E.M.) are shown below.

Rat	Treatment	Incubation	Rate constant (min^{-1})
—	—	ATP alone	-0.08 ± 0.03
Fed	Saline	Supernatant	-0.68 ± 0.10*
Fed	Cycloheximide	Supernatant	-0.60 ± 0.07*
Starved	Saline	Supernatant	-1.22 ± 0.10*†
Starved	Cycloheximide	Supernatant	-0.74 ± 0.08*‡

* $P < 0.001$ for effect of supernatant fraction; † $P < 0.01$ for effect of starvation; ‡ $P < 0.01$ for effect of cycloheximide; for other differences $P > 0.05$.

Correlation between activity of kinase/activator in rat heart mitochondria and the proportion of pyruvate dehydrogenase complex in the active form in the heart during refeeding of starved rats

The purpose of this experiment was to ascertain whether re-feeding of 48h-starved rats decreased the activity of kinase activator in mitochondrial supernatant fraction, and if so whether this correlated with the conversion of inactive complex into active complex, which is known to occur when starved rats are refed (Wieland *et al.*, 1973). If kinase/activator is an important factor in the conversion of active complex into inactive complex during starvation, then re-activation of complex induced by re-feeding should be correlated with disappearance of kinase/activator.

The results of this experiment are shown in Fig. 6. The activity of kinase/activator (expressed as the apparent first-order rate constant for ATP-induced

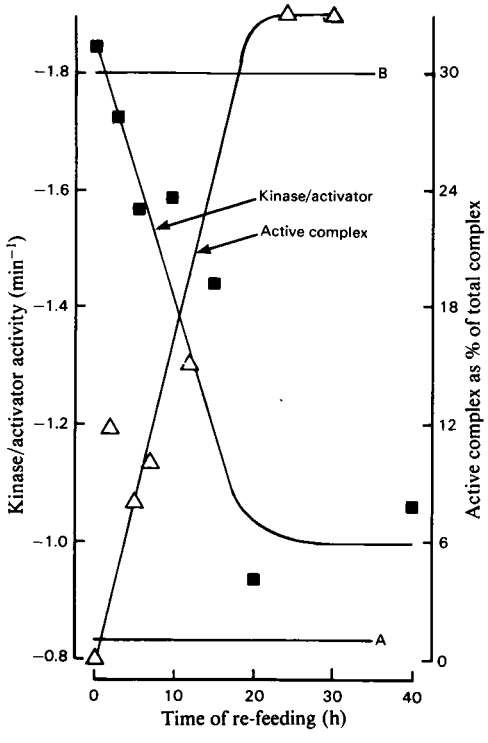

Fig. 6. *Effect of re-feeding of 48 h-starved rats on the activity of kinase/activator and the proportion of pyruvate dehydrogenase complex in the active form in rat heart*

Rats were re-fed after 48 h starvation, and hearts were removed at the times shown for assay of pyruvate dehydrogenase complex (△) and for assay of kinase/activator in the high-speed-supernatant fraction of mitochondrial extracts (■) (for further details see the Experimental section). Kinase/activator was assayed under conditions given in the legend to Fig. 5 and its activity is given as the apparent first-order rate constant for inactivation by ATP. Data are based on two mitochondrial preparations. Each point is the mean of ten observations (■) or four observations (△) respectively. The disappearance of kinase/activator and the appearance of active complex were linearly correlated ($r = -0.92$). (A) Kinase/activator in fed controls; (B) active complex in fed controls.

inactivation of pig heart complex) decreased within 20 h of re-feeding 48 h-starved rats, to approach that of fed controls. The decrease in activity of the kinase/activator in mitochondrial supernatant fraction was correlated with the concomitant increase in the heart in the proportion of complex in the active form and with the concentration of active complex (the total concentration of complex was not changed by re-feeding; results not shown). Analysis of the correlation by least-squares linear-regression analysis showed a significant linear negative correlation between (activity of kinase/activator) and (proportion of complex in the active form). The correlation coefficient (r) was −0.92 and deviations from linearity were not significant. Nevertheless the activity of kinase/activator was significantly greater than that of the fed control after 40 h (in Fig. 6) and after 1 week of re-feeding (results not shown), whereas the concentration of active complex returned to that of the fed control after 20 h of re-feeding (Fig. 6).

Kinase/activator in high-speed-supernatant fraction prepared from ox kidney mitochondria

The purpose of these experiments was to ascertain whether kinase/activator is present in mitochondria prepared from a bulk source. Ox kidney mitochondria were prepared as described by Fatania *et al.* (1981), and a high-speed-supernatant fraction was prepared as with rat heart mitochondria. Kinase/activator was detected with the assay based on unprocessed pig heart complex (rate constant $-0.32 \pm 0.04\,min^{-1}$; mean ± S.E.M. for five time intervals, three or four observations at each). Activity was non-dialysable (for 18 h at 4°C against 170 vol. of 30 mM-potassium phosphate/2 mM-EGTA/2 mM-dithiothreitol, pH 7.0) (rate constant after dialysis $-0.52 \pm 0.06\,min^{-1}$) and thermolabile (rate constant after heating at 100°C for 15 min $-0.06 \pm 0.06\,min^{-1}$). Kinase activator was destroyed by trypsin (rate constant $-0.05 \pm 0.01\,min^{-1}$). High-speed supernatant (2 ml, equivalent to 24 mg of mitochondrial protein) was applied to a column (25.5 cm × 1.9 cm diameter) of Sephadex G-100 (superfine grade; 72 ml) and eluted with 30 mM-potassium phosphate/2 mM-EGTA/1 mM-dithiothreitol (pH 7); 1.95 ml fractions were collected. Kinase/activator was eluted at or close to the void volume (peak of kinase/activator, fraction 10; peak of Blue Dextran, fractions 9/10). This could be compatible with kinase/activator being pyruvate dehydrogenase kinase (M_r 93000; Reed & Pettit, 1981). These results indicate that ox kidney mitochondria are a bulk source of kinase/activator.

Conclusions

The main purpose of the present study was to ascertain whether kinase/activator is likely to be of physiological significance, and if so to devise the means (bioassay) and to locate a bulk source of kinase/activator for purification and characterization. An assay has been developed using ATP-dependent inactivation of dilute solutions of purified complex. This assay is assumed to be based on dissociation of kinase/activator at low concentrations of the complex leading to greater dependence on added kinase/activator through a mass-action

effect. Kinase/activator appears to be a protein of high M_r (approx. 100000 or greater); it was non-diffusible on dialysis, thermolabile, inactivated by trypsin and excluded by Sephadex G-100 (superfine grade). The concentration of kinase/activator in rat heart mitochondria was increased by starvation; the increase in concentration induced by starvation was inhibited completely by cycloheximide and 50% by puromycin, and was reversed by re-feeding of starved rats. It has been shown that the activity of kinase/activator is increased in heart mitochondria in alloxan-diabetes (Kerbey & Randle, 1981). There appears to be sufficient evidence about the physiological and pathological significance of kinase/activator to warrant its purification and characterization. The present study has provided a suitable bioassay and a bulk source for purification. Current evidence is consistent with kinase/activator being pyruvate dehydrogenase kinase which is not bound to pyruvate dehydrogenase complex, but the possibility of a protein activator of the pyruvate dehydrogenase kinase reaction has not been excluded.

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