THERMOLABILE FACTOR ACCELERATES PYRUVATE DEHYDROGENASE KINASE REACTION IN HEART MITOCHONDRIA OF STARVED OR ALLOXAN-DIABETIC RATS

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1. Introduction

The PDH complex of animal tissues is converted into PDHP complex and inactivated by PDH kinase (utilizing MgATP) and reactivated by PDHP phosphatase [1]. The kinase and phosphatase reactions constitute a cycle with the proportions of PDH and PDHP complexes depending on their relative rates. Alloxan-diabetes or starvation in the rat markedly decrease the concentration of active PDH complex by conversion into inactive PDHP complex [2]. The mechanism by which this is brought about is not completely understood. There is evidence that diabetes and starvation activate the kinase reaction and inhibit reactivation by the phosphatase. The PDH kinase reaction is activated by increasing concentration ratios of ATP/ADP, $NADH/NAD^+$ and acetyl CoA/CoA [3–5]. Oxidation of lipid fuels in diabetes and starvation may increase the concentration ratio of acetyl CoA/CoA and thus activate the kinase reaction. However, changes in the concentrations of effectors of the PDH kinase reaction do not explain fully the decreased proportion of active PDH complex in heart mitochondria in diabetes and starvation [6,7]. Evidence for a further mechanism activating PDH kinase in extracts of heart mitochondria from diabetic or starved rats was given in [8]. This was a stable mechanism which persisted through isolation, incubation and extraction of mitochondria. Inhibition of reactivation by PDHP phosphatase (EC 3.1.3.43) may be due to multi-site phosphorylation, i.e., dependent on PDH kinase activity.

To characterise this further mechanism of PDH kinase activation in heart mitochondria in diabetes and starvation it was necessary to ascertain whether the factor responsible can be separated from the PDH complex. Evidence is given here that rat heart mitochondria contain a thermolabile non-dialysable factor, which can be separated from the PDH complex by high speed centrifugation, and which activates the PDH kinase reaction. The activity of this factor is increased in heart mitochondria of 48 h starved or diabetic rats.

Abbreviations: ATPase, adenosine triphosphatase; DTT, dithiothreitol; EGTA, ethanedioxybis (ethylamine)-tetra-acetate; PDH complex, pyruvate dehydrogenase complex (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3); PDHP complex, pyruvate dehydrogenase phosphate complex; PDH kinase, pyruvate dehydrogenase kinase (EC 2.7.1.99); TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone

2. Experimental

Sources of materials, rats and details of induction of diabetes and preparation of rat heart mitochondria are given in [8].

PDH complex in mitochondrial extracts was assayed spectrophotometrically by coupling to arylamine acetyltransferase [5]. Incorporation of ^{32}P from [γ-^{32}P] ATP into PDHP complex was assayed on paper squares [9].

Rat heart mitochondria (1–1.5 mg protein) were incubated for 10 min at 30°C in 0.5 ml KCl medium to convert PDHP complex into PDH complex, separated by centrifugation and frozen in liquid N_2 [5]. For preparation of high-speed supernatant, mitochondria were extracted by freezing (liquid N_2) and thawing (30°C) (×3) in 30 mM potassium phosphate/10 mM EGTA/1 mM TLCK/10 mM DTT/25 μg oligomycin B/ml (pH 7.0) (4 mg mitochondrial protein/ml). The extract was centrifuged at 150 000 × *g* for 90 min and the supernatant aspirated. For assay of PDH kinase

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activity mitochondrial pellets were extracted either with extraction buffer as above or with high speed supernatant (3 mg mitochondrial protein/ml in either case).

For assay of PDH kinase activity, mitochondrial extracts were warmed to 30°C (2 min) and reaction initiated by addition to 0.3 mM of ATP or [γ-^{32}P]ATP (~250 dpm/pmol). Samples were taken for assay of PDH complex activity or of [^{32}P]protein at times given in section 3. Corrections were applied for loss of PDH complex activity in incubations without ATP (<2.5%/min); for non-specific binding of ^{32}P to paper squares (by mixing extract and ATP^{32} at 0°C and applying immediately); and for apparent incorporation of ^{32}P into proteins of high speed supernatant (<6% of that in mitochondrial extracts). High speed supernatants were devoid of PDH complex activity. Under the conditions of assay PDHP phosphatase was inactive and ATPase activity (<2% of ATP hydrolysed/min) showed no significant differences between experimental groups as in [8]. Evidence that PDHP complex accounts for at least 95% of ^{32}P incorporation into protein in heart mitochondrial extracts is given in [8,10].

Frozen pellets of incubated heart mitochondria were prepared on day 1; high-speed supernatants were prepared and PDH kinase assays were performed on day 2. Activities of PDH complex and PDH kinase in frozen mitochondrial pellets are stable for at least 30 h. High speed supernatant could be stored at 0°C for at least 24 h without loss of activity. Measurements in all experimental groups for control against starved or control against diabetic at particular periods of incubation were obtained in single experiments. However not all periods of incubation were encompassed in a single experiment. Inactivation of PDH complex by ATP is therefore expressed in percentage terms of (residual activity)/(initial activity). Incorporation of ^{32}P into PDHP complex is expressed as (pmol ^{32}P)/(munit of PDH complex at zero time).

3. Results and discussion

PDH kinase activity in mitochondrial extracts was assayed here by monitoring both disappearance of active PDH complex and incorporation of ^{32}P into PDHP complex. This was important because of the possibility of inactivation of PDH complex by mechanisms other than phosphorylation even though apparently ATP dependent. Here, close parallelism was observed between the rate of inactivation of PDH complex and ^{32}P incorporation.

The activity of PDH complex was significantly enhanced (relative to controls) in extracts of heart mitochondria from 48 h starved or alloxan-diabetic rats. This was apparent in experiments in which disappearance of active complex (fig.1) or in which ^{32}P incorporation (fig.2) was measured. These results are qualitatively and quantitatively similar to those obtained in [8]. Inactivation of PDH complex in extracts of mitochondria from starved or diabetic rats was essentially complete within 2 min (fig.1) whereas incorporation of ^{32}P was not complete at this time (fig. 2) (full incorporation is in excess of 2 pmol/munit [11]). In rat heart complex [11] ^{32}P incorporation continues after inactivation is complete because of phosphorylation of 2 sites additional to the inactivating site.

A high speed supernatant fraction of heart mitochondrial extracts (devoid of PDH complex activity) increased the rate of inactivation of PDH complex by the PDH kinase reaction in unfractionated mitochondrial extracts. High speed supernatant fraction from mitochondria of starved or diabetic rats was more active in this respect than that of control rats (fig.1). In order to assess statistical significance apparent first-order rate constants for inactivation were calculated by least-squares linear regression analysis (table 1). The fit was good; the intercept was not significantly different from 100% and deviation from linearity was not significant (not given). This analysis showed that high speed supernatants from starved or diabetic rats enhanced PDH kinase activity in extracts of control mitochondria to a significantly greater extent than high speed supernatant from control mitochondria. High speed supernatant from control mitochondria did not enhance PDH kinase activity in extracts of starved or diabetic mitochondria (there was in fact a significant inhibition in extracts of starved mitochondria). There was no significant difference in PDH kinase activity between control mitochondria extracted with starved (or diabetic) high speed supernatants and starved (or diabetic) mitochondria extracted with control high speed supernatant. Further experiments (not given) showed that enhancement of PDH kinase activity was proportional to the concentration of high speed supernatant. These results may indicate that increased concentration of a factor present in high speed supernatant fraction of mitochondrial extract is responsible for enhanced PDH kinase activity in heart

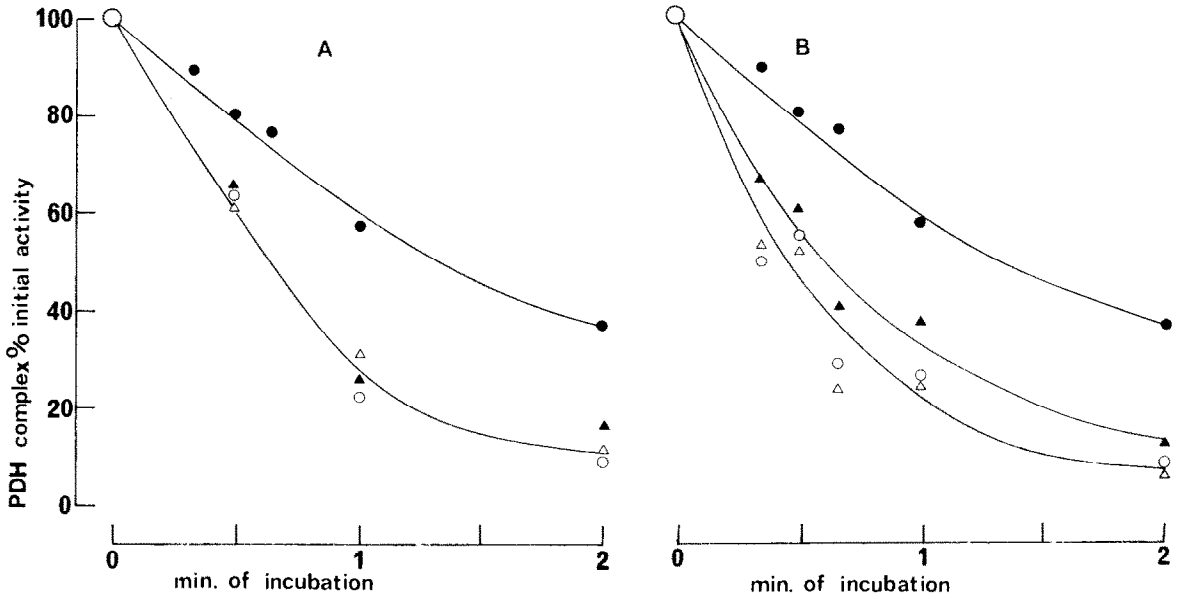

Fig.1. Inactivation of PDH complex by ATP in extracts of rat heart mitochondria. For details of incubation and methods used see legend to table 1 and section 2. (A) Buffer extracts of: (●) control; (○) starved; (▲) control mitochondria extracted starved high speed supernatant; (△) starved mitochondria extracted control high speed supernatant. (B) As (A) with diabetic in place of starved. Initial PDH complex activities (munits/mg mitochondrial protein; mean ± SEM of ⩾24 obs.) were, 71.3 ± 1.6 (control), 64.8 ± 3.4 (starved), 70.5 ± 2.2 (diabetic). For statistical significance of differences between regressions see legend to table 1. Data in (A) are based on 8 mitochondrial preparations (control and starved); data in (B) on 3 preparations (control and diabetics).

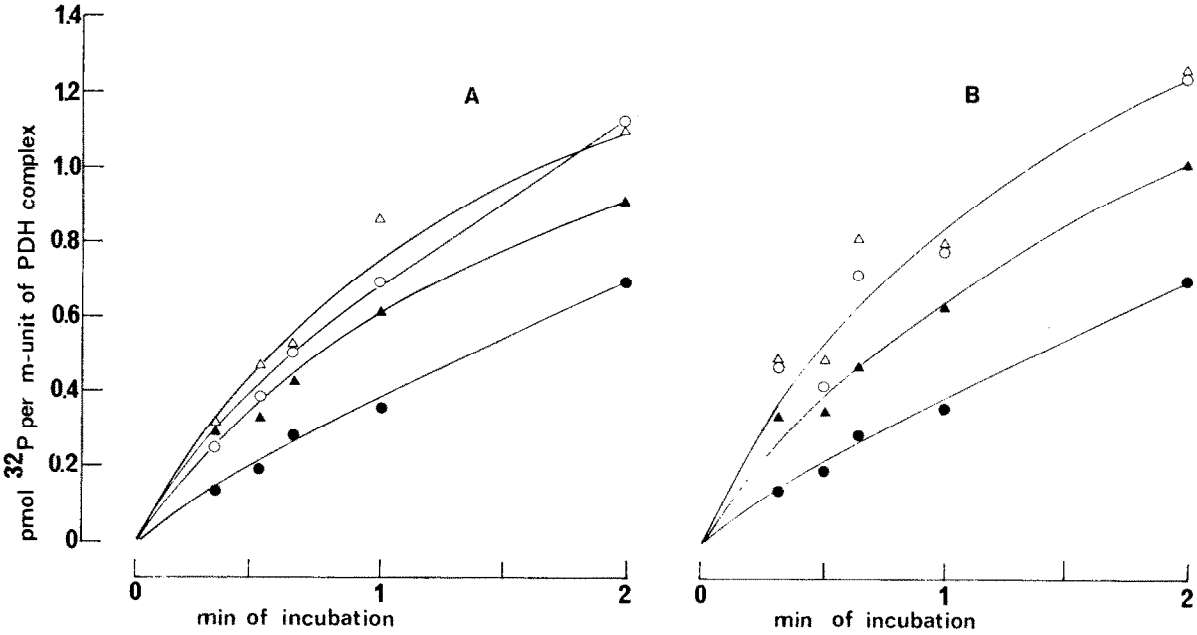

Fig.2. Incorporation of ^{32}P from [γ-^{32}P]ATP into PDH complex in extracts of rat heart mitochondria. Extracts of mitochondria prepared in extraction buffer or high speed supernatant were incubated with 0.3 mM [γ-^{32}P]ATP at 30°C and samples taken for assay of ^{32}P protein at times shown (for further details see section 2). (A) Buffer extracts of: (●) control; (○) starved; (▲) control mitochondria extracted starved high speed supernatant; (△) starved mitochondria extracted control high speed supernatant. (B) As (A) with diabetic in place of starved. Each point mean of ⩾6 obs. $P < 0.01$ at all time points for control against starved or diabetic in buffer extracts. For effect of starved high speed supernatant, $P < 0.01$ at 0.5 and 1 min, $P < 0.05$ at 0.33 min. For effect of diabetic high speed supernatant, $P < 0.01$ at 0.33, 0.5 and 1 min, $P < 0.05$ at 2 min. For other differences $P > 0.05$. Data in (A,B) are based on 3 mitochondrial preparations in each group (control and starved; control and diabetic).

Table 1
Inactivation of PDH complex by ATP in rat heart mitochondrial extracts; apparent first-order rate constants (min^{-1}; mean ± SEM)

High speed supernatant	Extracts of mitochondria from:		
	Control	Starved	Diabetic
None	−0.52 ± 0.03 (49)	−1.41 ± 0.07 (48)[a]	−1.15 ± 0.13 (16)[a]
Control	−0.73 ± 0.05 (50)[b]	−1.16 ± 0.06 (16)[a,b]	−1.32 ± 0.16 (16)[a]
Starved	−1.14 ± 0.09 (47)[a,b]	–	–
Diabetic	−1.01 ± 0.07 (16)[a,b]	–	–

[a] $P < 0.01$ for effects of starvation or diabetes; [b] $P < 0.01$ for effect of high speed supernatant; for other differences $P > 0.05$

Extracts of mitochondria (135 μl) prepared in extraction buffer or high speed supernatant were incubated with 0.3 mM ATP at 30°C and samples for assay of PDH complex taken at times shown in fig.1 (see section 2 for further details). Rate constants calculated by least squares linear regression analysis of (ln per cent zero time activity) against time. Number of obs. in parentheses

mitochondrial extracts of 48 h starved or alloxandiabetic rats.

These findings are borne out by measurements of incorporation of ^{32}P from [γ-^{32}P]ATP into PDHP complex shown in fig.2. The statistical significance of differences was established by Student's *t*-test at individual time points as ^{32}P incorporation did not closely conform to the first order rate equation.

The factor in high speed supernatant of starved mitochondria which enhanced inactivation of PDH complex by the PDH kinase reaction in extracts of control mitochondria, was inactivated by heating the supernatant at 100°C for 15 min and centrifuging to remove denatured protein. The results (% of initial PDH complex after 1 min with ATP; mean ± SEM for 5 obs.) were control (buffer extract) 49.9 ± 2.1, starved (buffer extract) 17.9 ± 0.7, control (extracted starved high speed supernatant) 21.0 ± 0.8, control (extracted boiled supernatant) 59.9 ± 1.26. Dialysis of high speed supernatant of starved mitochondria for 17 h at 4°C against 200 × vol. extraction buffer lacking oligomycin did not lead to loss of activity. The results (mean ± 0.5 difference for 2 obs.) were control (buffer extract) 74.8 ± 6 starved (buffer extract 21.9 ± 2.5, control extracted starved high speed supernatant, undialysed 25.2 ± 0, control extracted dialysed supernatant 28.9 ± 0.5. The factor is thus thermolabile and non-dialysable.

This study may show that the factor responsible for enhanced PDH kinase activity in extracts of heart mitochondria of 48 h starved or alloxan-diabetic rats can be separated from PDH complex by high speed centrifugation. The study shows further that the factor is thermolabile and non-dialysable. The factor may be an activator of the PDH kinase reaction or PDH kinase extrinsic to the PDH complex and separated from it by high-speed centrifugation. Because PDH complex in mitochondrial extracts loses activity during high-speed centrifugation it was not possible to make a valid comparison of PDH kinase activities in the sedimented PDH complexes. However there were no obvious differences of PDH kinase activity in complexes purified from hearts of control, starved or diabetic rats [12].

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