STARVATION OF LACTATING RATS LEADS TO ALTERATIONS IN THE BEHAVIOUR OF PYRUVATE DEHYDROGENASE KINASE WHICH PERSIST IN THE SEMI-PURIFIED PYRUVATE DEHYDROGENASE COMPLEX OF THE MAMMARY GLAND BUT ARE PARTLY REVERSIBLE IN VITRO

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Received 6 November 1978

1. Introduction

The mitochondrial PDH complex of lactating rat mammary gland, in common with the analogous enzyme in other tissues is regulated by a phosphorylation–dephosphorylation cycle [1]. The $MgATP^{2-}$ requiring kinase, which phosphorylates and inactivates the α-subunit of the PDH complex, is tightly bound to and co-purifies with the PDH complex [2,3].

We have reported [4,5] that 24 h starvation leads to increased steady-state phosphorylation of PDH in vivo and this is at least partly due to two very marked changes in the behaviour of the PDH kinase when assayed in tissue or mitochondrial extracts:

(1) An enhanced activity of the enzyme;
(2) A resistance to pyruvate inhibition when compared to the enzyme from fed animals.

We have now shown that these changes persist through a multi-step partial-purification of the PDH complex. Further studies on the complex from starved animals have shown that when freshly prepared the PDH kinase has reduced rather than absent sensitivity to pyruvate inhibition (the K_i for pyruvate was increased by a factor of ~100). Sensitivity to pyruvate inhibition could be restored during incubation in vitro by a time- and temperature-dependent process which was unaffected by inhibitors of proteolysis or of dephosphorylation. Under conditions when pyruvate sensitivity was restored, the PDH kinase from starved animals still showed enhanced activity in the absence of pyruvate. Apparently, there is no necessary link between enhanced PDH kinase activity in the absence of pyruvate and diminished sensitivity of the PDH kinase to pyruvate inhibition.

Abbreviations: PDH, pyruvate dehydrogenase enzyme complex; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone

2. Methods

Materials and enzyme assays were generally as in [4,5]. Polyethylene glycol 6000 and TLCK were from Sigma London, Norbiton Station Yard, Surrey KT2 7BH. Modifications of procedures described before are indicated in the figure legends.

Purification of lactating rat mammary gland PDH was carried out by combining the procedures in [1,6]. The yields after each step of the purification are given in parentheses as a percentage of the total PDH activity originally present after homogenization of the mammary glands. These percentages are stated as the mean ± SEM of 8 separate preparations.

Fresh or stored (−17°C for 1 month) glands, 6–8 pairs, were homogenized for 5 min at 4°C in 30 mM triethanolamine, 1 mM dithiothreitol, pH 7.0 (6.3 mg/g wet wt) using an Atomix blender. The homogenate was centrifuged at 21 000 × *g* for 30 min at 4°C and the precipitate discarded. The supernatant was filtered through muslin and then contained 90–130 units total PDH activity and 5000–6000 mg soluble protein in 300–400 ml (representing PDH spec. act. ~19 mUnits/mg protein). While the supernatant was kept at 4°C and vigorously stirred, $CaCl_2$

was added to final conc. 10 mM. The pH was restored to 7.0 with 10% (v/v) acetic acid. The precipitate of calcium caseinate was removed by centrifugation at 21 000 × *g* for 20 min at 4°C and the supernatant retained (72.7 ± 2.7%). This supernatant was warmed to 20°C and stirred while lowered to pH 6.5 with 10% (v/v) acetic acid. NaCl, 5 N (0.01 vol.) was then added followed by 0.05 vol. 50% (w/v) polyethylene glycol. The solution was centrifuged at 21 000 × *g* for 20 min at 20°C, the precipitate discarded and the supernatant retained (55.6 ± 2.8%). To this supernatant a further 0.05 vol. 50% (w/v) polyethylene glycol was added. After centrifugation at 21 000 × *g* for 20 min at 20°C the small precipitate was distributed in 25 ml cold 30 mM triethanolamine, 1 mM dithiothreitol (pH 7.0). Undissolved material was removed by centrifugation at 32 000 × *g* for 10 min at 4°C, the supernatant (33.6 ± 2.8%) was adjusted to pH 6.3 with 1% (v/v) acetic acid and the centrifugation repeated. The supernatant was decanted (17.5 ± 1.9%) and lowered to pH 5.6 by further addition of 1% (v/v) acetic acid. The precipitated PDH was collected by centrifugation at 32 000 × *g* for 15 min at 4°C. The small precipitate was resuspended in the ice-cold triethanolamine, dithiothreitol buffer (9.2 ± 1.2%). This solution was readjusted to pH 7.0 with 0.1 N potassium hydroxide. The resuspended PDH was then diluted to total PDH act. 6.1 ± 0.7 (8) Units/ml. During the course of the preparation the percentage total activity of the enzyme (the percentage of PDH in the dephosphorylated form) gradually increased until the complex was completely activated.

This purification procedure yielded 30–50 mg soluble protein and PDH spec. act. 0.3–0.5 Units/mg.

3. Results and discussion

The enhanced PDH kinase activity, shown [4] in whole tissue and mitochondrial extracts of lactating rat mammary gland from 24 h starved rats, persists through a multi-step semi-purification of the PDH complex (fig.1). However, the reduced pyruvate sensitivity of this PDH kinase, shown in vivo [5] and in vitro [4,5] after 24 h starvation, could only be observed in preparations involving freshly-extracted mammary glands (compare fig.1A with fig.1B). Storage at −17°C for 1 month apparently led to

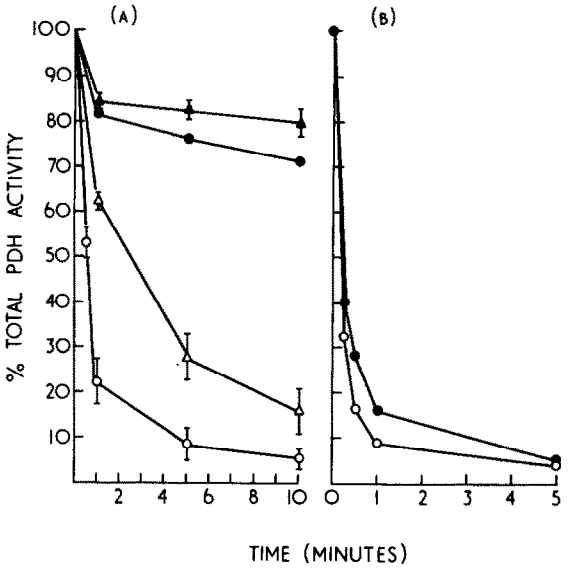

Fig.1. Effect of pyruvate on ATP inactivation of semi-purified PDH from lactating mammary glands of fed and starved rats. PDH (5–8 Units/ml) was preincubated in 30 mM triethanolamine–HCl, 1 mM dithiothreitol (pH 7.0) with 0.1 mM $MgCl_2$ for 5 min at 30°C. EGTA, 5 mM, was added and the incubation continued for a further 2 min with or without the addition of 1 mM sodium pyruvate. ATP, 5 mM, was then added. Samples were transferred at the indicated times to cuvettes containing assay medium at room temperature. After warming to 30°C the PDH activity in each cuvette was determined. The reaction was initiated by addition of CoA. 'Total PDH activity' is the activity of the totally dephosphorylated enzyme in the experiment before the addition of ATP. (△–△) PDH from fed rats incubated with 5 mM ATP. (▲–▲) PDH from fed rats incubated with 1 mM sodium pyruvate and 5 mM ATP. (○–○) PDH from 24 h starved rats incubated with 5 mM ATP. (●–●) PDH from 24 h starved rats incubated with 1 mM sodium pyruvate and 5 mM ATP. (A) PDH prepared from glands stored at −17°C for 1 month. Points drawn with standard error bars indicate mean ± SEM of 3 separate preparations. Other points are means of 2 separate preparations. (B) PDH prepared from glands removed from animals immediately before commencing preparation. A second fresh preparation behaved similarly.

restoration of pyruvate sensitivity of the PDH kinase. Pyruvate sensitivity could also be restored to the PDH kinase of 24 h starved animals in vitro (fig.2). To prevent restoration of sensitivity to pyruvate inhibition of PDH kinase semi-purified PDH was stored at −196°C in liquid nitrogen. This was sufficient to preserve for 7 days the total activity of PDH and the

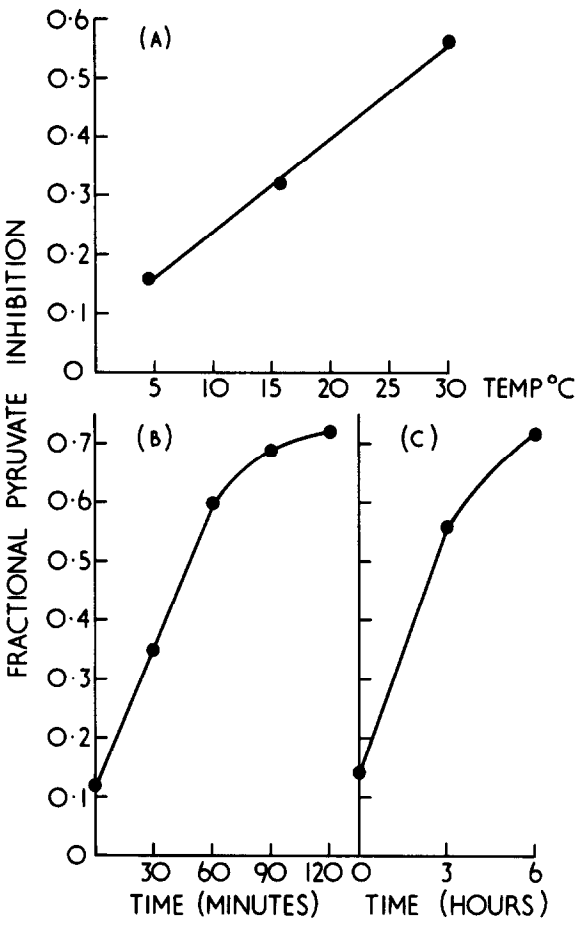

Fig.2. Effect of time and temperature of incubation on pyruvate inhibition of ATP inactivation of mammary gland PDH from 24 h starved lactating rats. PDH (5.8 Units/ml) in 30 mM triethanolamine, 1 mM dithiothreitol (pH 7) was incubated for stated times and temperatures. Then 0.1 mM $MgCl_2$, 5 mM EGTA, 5 mM ATP were added in that order. When 1 mM sodium pyruvate was required to be present it was added before the ATP. The time courses of inactivation with and without pyruvate were then determined as described in the legend to fig.1:

$$\text{Fractional pyruvate inhibition} = \frac{(a-a_1) - (a-a_2)}{(a-a_1)}$$

where a = PDH activity before exposure to ATP, a_1 = PDH activity after 30 s exposure to ATP, a_2 = PDH activity after 30 s exposure to ATP in the presence of pyruvate. During all these incubations there was no change in the total PDH activity, nor were there any changes in the time course of inactivation by ATP in the absence of pyruvate. (A) Changes in fractional pyruvate inhibition after incubation for 60 min at indicated temperatures. (B) Changes in fractional pyruvate inhibition at 30°C. (C) Changes in fractional pyruvate inhibition at 4°C. Data are means of 2 separate preparations.

characteristic properties of PDH kinase from glands of starved animals.

The increase in fractional pyruvate inhibition (defined in fig.2 legend) during incubation is a time- and temperature-dependent process (fig.2). Incubation of PDH at 30°C for 60 min as in fig.2 legend resulted in an increase of the fractional pyruvate inhibition to 0.72. Addition to the incubation medium of 1 mM TLCK (an inhibitor of tryptic proteolysis [7]), 20 mM sodium fluoride or 100 mM potassium phosphate (inhibitors of phosphoprotein phosphatases [8–10], had no effect on the increase in fractional pyruvate inhibition. Total PDH activity (i.e., the activity of the totally dephosphorylated enzyme) and PDH kinase activity in the absence of pyruvate were unaffected by any of the described incubations.

This data suggests that the in vitro restoration of pyruvate sensitivity of PDH kinase from 24 h starved animals was not due to nonspecific proteolysis or dephosphorylation at least by a sodium fluoride or phosphate sensitive phosphoprotein phosphatase.

The in vitro recovery of pyruvate sensitivity of PDH kinase without any alteration in PDH kinase activity in the absence of pyruvate suggests that these two changes in properties of PDH kinase after 24 h starvation are not necessarily connected although in vivo they could both contribute to maintenance of increased α-subunit phosphorylation.

Measurement of the K_i of pyruvate for PDH kinase in preparations of lactating rat mammary gland from fed (1 prep.) and starved (1 prep.) gave 0.07 mM and 7 mM, respectively. If the inhibitory effect of pyruvate on the PDH kinase reaction is achieved by steric interference with the α-subunit/PDH kinase interaction starvation may diminish the effectiveness of pyruvate by changing the orientation of enzyme to substrate. We have considered the possibility that a labile effector is involved in preventing pyruvate inhibition of PDH kinase but it is difficult to understand persistence of such an effector through the purification process which involved periods of at least 60 min at 20°C. A further complicating feature which we have noted is that the calcium caseinate precipitation step was important in promoting PDH kinase activity in the final semi-purified complex whether derived from glands of fed or starved animals. We regard these observations as pointing to a set of complex interactions whose definition will require a more

direct approach to investigate the relative amounts and orientation of the components of PDH.

Acknowledgement

M.A.B. holds a research studentship with the Medical Research Council. The Council has also assisted with other expenses involved in this work.

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