ENHANCED ACTIVITY OF PYRUVATE DEHYDROGENASE KINASE IN RAT HEART MITOCHONDRIA IN ALLOXAN-DIABETES OR STARVATION

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1. Introduction

The PDH complex of animal tissues is converted to PDHP complex and inactivated by an intrinsic PDH kinase (utilizing $MgATP^{2-}$) 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 PDH complex without changing the total concentration (PDH + PDHP) [2]. The mechanism by which this is brought about is not completely understood. The PDH kinase reaction is activated by increasing concentration ratios of ATP/ADP, $NADH/NAD^+$ and acetyl CoA/CoA and inhibited by pyruvate [1,3,4]. However, changes in the concentrations of these effectors do not explain the decreased proportion of PDH complex in heart mitochondria in diabetes [5,6]. Reactivation of PDH complex by PDHP phosphatase is inhibited in heart mitochondria of starved or diabetic rats [7]. This inhibition may be due to the phosphorylation of sites additional to an inactivating site in the PDH complex [7,8] and is therefore dependent on the PDH kinase reaction. Evidence is given here that the PDH kinase reaction is accelerated in heart mitochondria from alloxan-diabetic or 48 h starved rats by a novel mechanism which does not appear to depend on known metabolite effectors of PDH kinase.

Abbreviations: DTT, dithiothreitol; EGTA, ethanedioxybis (ethylamine)-tetra-acetate; PDH, pyruvate dehydrogenase; PDHP, pyruvate dehydrogenase phosphate. PDH complex is (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3); ATPase, adenosine triphosphatase; SDS, sodium dodecyl sulphate

2. Experimental

2.1. *Materials*

Nagarse was from Hughes and Hughes Ltd., Romford, Essex. Sources of all other reagents and of animals, and details of induction of alloxan-diabetes are given in references [5–7]. Pig heart [^{32}P]PDHP complex (1.7 nmol P/unit PDH complex) was prepared as in [8]. One unit of enzyme activity is 1 μmol substrate utilized or product formed/min at 30°C.

2.2. *Methods*

PDH complex in mitochondrial extracts was assayed spectrophotometrically by coupling to arylamine acetyltransferase [5]. ATPase was assayed by release of $^{32}P_i$ from [γ-^{32}P]ATP [9]. Details of SDS polyacrylamide disc gel electrophoreses are given in [10].

Rat heart mitochondria were prepared with the bacterial protease Nagarse [11] because this method gave minimal contamination with oligomycin-insensitive ATPase. Mitochondria (1.1 mg protein) were incubated for 10 min at 30°C in KCl medium without respiratory substrate (to convert PDHP complex into PDH complex [5]) separated by centrifugation and frozen [5]. The pellets were extracted by freezing in liquid N_2 and thawing at 30°C (× 3) in 30 mM potassium phosphate/10 mM EGTA/1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone/5 mM DTT/25 μg/ml oligomycin/pH 7.0 (2.75 mg mitochondrial protein/ml). PDH kinase was assayed in these extracts by the decline in activity of PDH complex and by formation of ^{32}P-protein [12]. After assay of zero time PDH complex, extracts (600 μl) were preincubated for 2 min at 30°C and the PDH kinase

reaction initiated with 18 μl of 10 mM [γ-^{32}P]ATP (45 μCi/μmol). Samples were taken for assay at times shown in the figures.

Magnesium required for the kinase reaction (as $MgATP^{2-}$) was derived solely from the mitochondria and concentrations (in ng-atoms Mg/ml of extract) were 90 ± 12 (control) and 80 ± 5.3 (diabetic) (mean ± SEM for at least six determinations by atomic absorption spectrometry). The computed concentration of $MgATP^{2-}$ [see 9] was approximately 50 μM. It was necessary to work at low concentrations of Mg^{2+} (computed as 5 μM) to minimise activities of PDHP phosphatase (which requires Mg^{2+}) and ATPase. With these conditions, PDHP phosphatase was totally inactive; there was no detectable loss of protein-bound-^{32}P on incubation of 0.2 unit [^{32}P]PDHP with 200 μl of mitochondrial extract for 9 min at 30°C. ATPase removed 1–1.5% of ATP/min. Under these conditions there were no significant differences in PDHP phosphatase or ATPase activities between extracts from control, diabetic or starved animals. Extracts incubated without ATP showed no more than 10% loss of PDH activity over 6 min.

3. Results and discussion

The PDH kinase activity was significantly enhanced (relative to controls) in extracts of heart mitochondria from alloxan-diabetic or 48 h starved rats. This was apparent in the more rapid initial rates of inactivation of PDH complex (fig.1, panels a, b) and of incorporation of ^{32}P into protein (fig.1, panels c, d). With all extracts, inactivation of PDH complex was essentially complete in 6 min (fig.1) whereas ^{32}P incorporation continued beyond this time (not shown). These properties of the PDH kinase reaction in mitochondrial extracts are very similar to those observed with purified pig heart PDH complex [10]. SDS polyacrylamide disc gel electrophoresis of extracts (control and diabetic) after 30 min of incubation with [γ-^{32}P]ATP showed only a single band of radioactivity with the same mobility as the ^{32}P-labelled α-subunit of purified pig heart [^{32}P]PDH complex (not shown). This is consistent with other evidence that this is the only major peptide in which phosphorylation can be detected in isolated rat heart mitochondria [7,13].

The PDH kinase reaction is activated by NADH and acetyl CoA and inhibited by NAD^+, CoA and ADP [1,3-6]. It was important to investigate the possibility that the difference in kinase activities between extracts of control and diabetic mitochondria might be due to differences in the concentration ratios of ATP/ADP, NADH/NAD^+ and acetyl CoA/CoA. The mitochondrial contents of ATP and ADP [6] are less than 2% of the ATP added to initiate the kinase reaction in the present study and, moreover, no difference of significance between control and diabetic mitochondria was observed [6]. The mitochondrial contents of NADH and acetyl CoA after no substrate incubation are too low for reliable assay even by sensitive methods. Such estimates as are available [6] suggest that the concentration ratios in the present

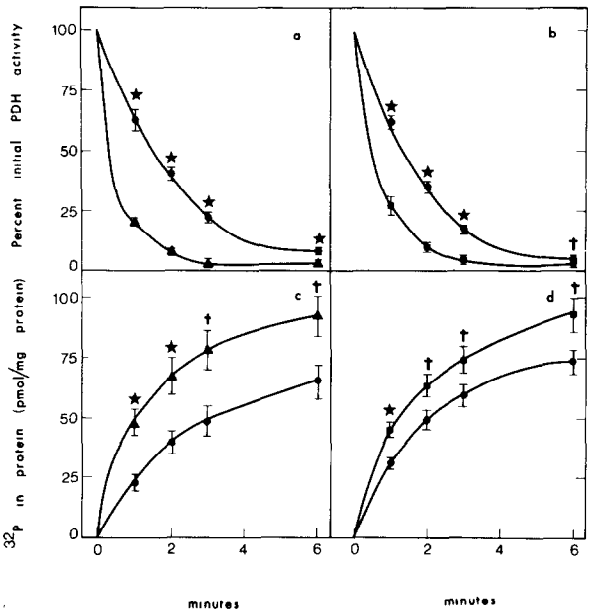

Fig.1. PDH kinase activity in extracts of heart mitochondria from control (●), alloxan-diabetic (▲), and 48 h starved (■) rats. Extracts were prepared as described in experimental section and pre-incubated at 30°C for 2 min. The PDH kinase reaction was initiated by adding [γ-^{32}P]ATP to 0.3 mM. Initial PDH complex activities (mean ± SEM in mU/mg protein) were, in panels (a) and (c) 101 ± 7.6 (control), 88 ± 7.3 (diabetic); in panels (b) and (d) 109 ± 3.9 (control), 106 ± 4.6 (starved); means based on not less than 4 observations. Points shown as mean ± SEM. For differences (control vs. diabetic) or (control vs. starved) $^*P<0.01$; $^+P<0.05$.

kinase assays would be no greater than 0.15 μM NADH/10 μM NAD^+ and 0.36 μM acetyl CoA/3.22 μM CoA. The actual ratios are likely to be much lower as mitochondrial extracts oxidise NADH and hydrolyse acetyl CoA. The rates of the kinase reaction in control and diabetic mitochondria were not altered by addition of CoA (to 100 μM added CoA) plus NAD^+ (to 800 μM added NAD^+), or of oxaloacetate (to 100 μM) during preincubation and incubation. Activation of the bovine kidney and pig heart PDH kinase reaction by NADH or acetyl CoA is reversed by NAD^+ or CoA [3–5]. The mitochondrial extracts in the kinase assays in this paper contained citrate synthase (3 U/ml) and malate dehydrogenase (8 U/ml). Addition of oxaloacetate should therefore lead to removal of acetyl CoA (as citrate) and of NADH (by oxidation). It seems unlikely therefore that the enhanced kinase activity in extracts of heart mitochondria from diabetic (or starved) rats is due to the action of known metabolite effectors of the kinase.

The PDH kinase reaction is inhibited by pyruvate [1,9] and pyruvate increases the proportion of PDH complex in heart mitochondria [5–7]. The effect of pyruvate in heart mitochondria is markedly reduced by alloxan-diabetes or starvation [5–7]. Pyruvate (2.7 mM) inhibited the PDH kinase reaction in extracts of rat heart mitochondria (compare panels bearing the same letter code in figs.1 and 2). The enhanced activity of the kinase reaction (relative to controls) in extracts of mitochondria from diabetic or starved rats was retained in the presence of 2.7 mM pyruvate (fig.2, panels a–d).

The mechanism of the enhanced PDH kinase activity in heart mitochondria in diabetes and starvation shown in these studies is not known. The mechanism must involve rather stable factor(s) because it persists through isolation, incubation and extraction of mitochondria. Possibilities include increased concentration of PDH kinase in the PDH complex or activation of the kinase reaction by covalent modification of the complex. Acetylation or reduction of lipoyl residues in the complex, which have been reported to activate the PDH kinase reaction [14], are unlikely to be involved here as the kinase activation observed in this study was not reversed by addition of CoA + NAD^+. The significance of this activation of the kinase in diabetes and starvation may be twofold. It may facilitate inactivation of the complex

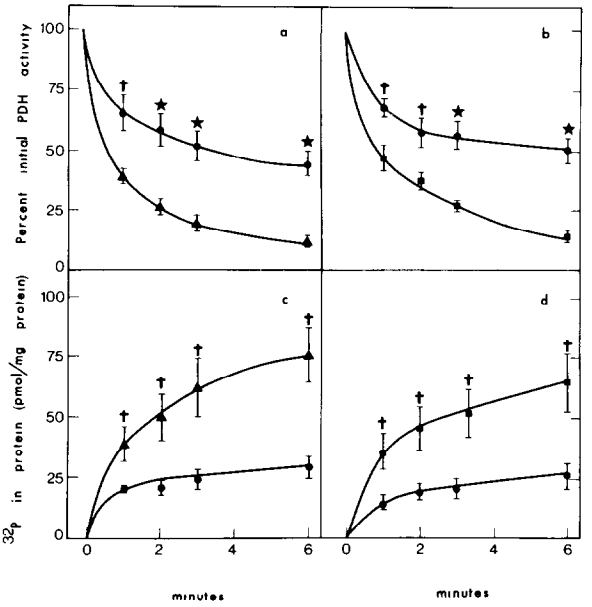

Fig.2. PDH kinase activities in extracts of heart mitochondria from control (●), alloxan-diabetic (▲), and 48 h starved (■) rats. Details of incubation as in fig.1 except that sodium pyruvate (2.75 mM) was present throughout pre-incubation and incubation. Initial PDH complex activities were, in panels (a) and (c) 108 ± 7.6 (control), 91 ± 7.7 (diabetic); in panels (b) and (d) 103 ± 11.3 (control), 71 ± 7.2 (starved); means based on not less than 4 observations. Points shown as mean ± SEM. For differences (control vs diabetic) or (control vs starved) $^*P<0.01$; $^+P<0.05$.

via phosphorylation of an inactivating site as shown in this study. It may also inhibit reactivation of PDHP complex by PDHP phosphatase by facilitating the phosphorylation of sites additional to the inactivating site [8] if rat heart PDH complex is similar to pig heart PDH complex.

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References

[1] Linn, T. C., Pettit, F. H., Hucho, F. and Reed, L. J. (1969) Proc. Natl. Acad. Sci. USA 64, 227–234.

[2] Wieland, O. H., Siess, E. A., Schulze-Wethmar, F. H., von Funcke, H. and Winton, D. (1971) Arch. Biochem. Biophys. 143, 593–601.

[3] Pettit, F. H., Pelley, J. W. and Reed, L J. (1975) Biochem. Biophys. Res. Commun. 65, 575–582.

[4] Cooper, R. H., Randle, P. J. and Denton, R. M. (1975) Nature (London) 257, 808–809.

[5] Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. and Denton, R. M. (1976) Biochem. J. 154, 327–348.

[6] Kerbey, A. L., Radcliffe, P. M. and Randle, P. J. (1977) Biochem. J. 164, 509–519.

[7] Hutson, N. J., Kerbey, A. L., Randle, P. J. and Sugden, P. H. (1978) Biochem. J. in press.

[8] Sugden, P. H., Hutson, N. J., Kerbey, A. L. and Randle, P. J. (1978) Biochem. J. 169, 433–435.

[9] Cooper, R. H., Randle, P. J. and Denton, R. M. (1974) Biochem. J. 143, 625–641.

[10] Sugden, P. H. and Randle, P. J. (1978) Biochem. J. in press.

[11] Chappell, J. B. and Hansford, R. G. (1978) in: Subcellular Components, 2nd edn, (Birnie, G. D., ed) p. 77, Butterworths, London.

[12] Corbin, J. D. and Reimann, E. M. (1974) Methods Enzymol. 38, 287–299.

[13] Hughes, W. A. and Denton, R. M. (1976) Nature (London) 26, 471–473.

[14] Cate, R. L. and Roche, T. E. (1978) J. Biol. Chem. 253, 496–503.