ON THE MECHANISM OF IRREVERSIBLE PYRUVATE DEHYDROGENASE INACTIVATION IN LIVER MITOCHONDRIAL EXTRACTS

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1. Introduction

During earlier studies on pyruvate dehydrogenase (PDH) (EC 1.2.4.1) interconversion in animal tissues we had observed that the activity of the enzyme, quite different from liver homogenates, declined rapidly and irreversibly in extracts prepared from isolated rat liver mitochondria. Complete preservation of PDH activity in the mitochondrial extracts was achieved by addition of a particle free rat liver supernatant (RLS) [1,2]. These findings which could not be related to inhibition or activation by enzyme phosphorylation and dephosphorylation, respectively prompted us to study this problem in more detail. The data reported here suggest that limited proteolysis probably by some lysosomal enzyme(s) is responsible for the inactivation of the PDH complex.

2. Experimental

Mitochondria were prepared from the livers of normally fed Wistar rats as described [1]. The particles obtained from two livers were suspended in 2 ml 0.25 M sucrose–0.1 mM EDTA, pH 7.2. Prior to extraction one vol of the mitochondrial suspension was mixed with 4 vol of a solution consisting of 90 mM KCl, 30 mM Tris, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM KH_2PO_4, and 5 mM sodium pyruvate, brought to pH 7.2 with 0.1 M HCl, and incubated for 3 min at 25°C. By this treatment PDH is activated due to conversion of the inactive to the active form [1]. After centrifugation (at 4°C) the particles were washed once with ice-cold buffer solution of the same composition but pyruvate omitted, and recentrifuged. The sediments were frozen in liquid nitrogen and thawed (twice), and homogenized 10 sec with 5 mM potassium phosphate buffer pH 7.0 with an Ultra-Turrax disintegrator (Fa. Janke u. Kunkel KG, Staufen/Br., Germany) in an ice bath. The extracts containing about 5 mg protein/ml could be stored in the deep freezer for 3 days without change in PDH-activity. PDH was measured photometrically at 405 nm with *p*-nitroaniline and arylamine acetyltransferase [13] by the microassay described in [4]. Protein was determined by the Biuret method after removal of lipids [5]: An appropriate volume of mitochondrial extract or suspension (not more than 0.2 ml) is deproteinized by addition of 1 ml dioxane and centrifuged. The dioxane is removed by suction and the precipitate is washed with 1 ml 0.3 M TCA, and again centrifuged. The precipitate is then dissolved in 1 ml of biuret reagent, and the extinction measured as usually at 546 nm.

Other enzymes as well as coenzymes and substrates were products of Boehringer Mannheim GmbH (Germany). Usual chemicals were of analytical grade from E. Merck (Darmstadt, Germany). Bovine serum albumin was from Behringwerke AG, Marburg, Germany, Trasylol® from Bayer AG, Leverkusen, Germany.

3. Results and discussion

The time course of PDH inactivation during incubation of mitochondrial extract is illustrated in fig.1. As may be seen there is only little inactivation at 0°C whereas, at 25°C, 50% of enzyme activity has disappeared within less than 10 min. Fig.1 furthermore shows that addition of native rat blood serum (RBS) comple-

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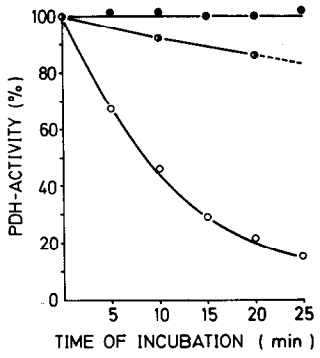

Fig.1. Time-dependent inactivation of the pyruvate dehydrogenase complex in rat liver mitochondrial extracts. 500 μl samples of RLM extract with an initial PDH content of 25–30 mU were incubated at 25°C (o———o) and at 0°C, respectively (•———•), and at 25°C in the presence of RBS corresponding to 200 μg protein/ml (•———•). At the times indicated 50 μl samples were removed for PDH determination as described in section 2.

tely abolishes PDH inactivation. The stabilizing effect of RBS as a function of protein concentration is shown in fig.2. Accordingly, about 25 μg protein/ml, e.g. a

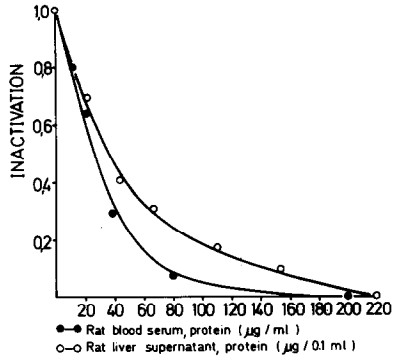

Fig.2. Effect of RLS and RBS on PDH inactivation as a function of protein concentration. Samples of RLM extracts were incubated at 25°C with the addition of RBS or RLS corresponding to the protein concentrations given in the abscissa. 50 μl samples were removed at 5 and 30 min, respectively for PDH determination. PDH-inactivation as calculated from the decrease in PDH activity during the 5–30 min interval is expressed relative to control values without added stabilizing proteins. RLS was prepared by homogenization of fresh rat liver with isotonic sucrose medium and centrifugation for 1 hr at 150 000 *g* at 2°C, as described [10].

twothousand fold dilution of native RBS was effective in half maximal inhibition of PDH inactivation. By comparison, rat liver supernatant (RLS), which also stabilizes PDH is more than ten times less effective than RBS. 24 hr dialysis of RBS did not influence its stabilizing effect whereas this was lost completely upon boiling for 5 min. Neither bovine serum albumin (up to 0.8%) nor Trasylol® (1000 U/ml) protected PDH from inactivation. Whereas Lubrol PX (10 mM) showed only a slight stabilizing effect, inactivation was reduced by about 50% in the presence of 2% Triton X-100. A similar protective effect on PDH activity was obtained with human α_2-macroglobulin (kindly supplied by Dr Fritz, Institut für Klinische Chemie und Klinische Biochemie, Munich, Germany) (200 μg/ml). The experiments so far suggest that the observed inactivation of PDH may be due to enzymatic degradation probably by some protease(s) (tentatively called 'inactivase') present in the mitochondrial extracts. The occurrence of inactivase-inhibitor(s) in RBS or RLS, for instance α_2-macroglobulin, would then explain the stabilizing effects of these preparations.

Since conventionally prepared liver mitochondria always contain lysosomes, experiments were carried out using mitochondria which had been depleted from lysosomal activity by digitonin treatment [6]. As illustrated in fig.3 PDH remains fully stable when extracts from such lysosome-depleted mitochondria are incubated at 25°C. On the other hand, as also shown in fig.3, inactivation is observed again if an extract obtained from purified rat liver lysosomes is added to the incubations. This seems to indicate that the 'inactivase' activity of RLM extracts stems from accompanying lysosomes. The rate of PDH inactivation was greatest at pH 6.5 and declined markedly in the alkaline range. Unfortunately the range below pH 6.5 could not be assayed owing to isoelectric precipitation of PDH at lower pH values.

In order to see whether the two molecular forms of PDH might behave differently as substrates for the 'inactivase', extracts were used from mitochondria which had not been pre-treated for activation and thus contained both the phosphorylated and the dephosphorylated species of the enzyme. As shown in table 1 there is a proportional decrease of PDH-activities either determined before (= PDH_a) or after conversion of the inactive portion of the enzyme into the active form (= PDH total) by treatment with PDH-phosphatase [8]. Thus

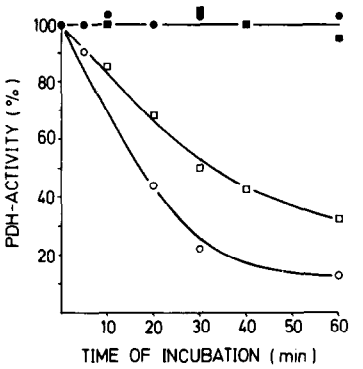

Fig.3. Lack of PDH inactivation in extracts from lysosome-depleted rat liver mitochondria. For the experiments indicated by circles mitochondria originating from the same homogenate were prepared either by the conventional procedure (open circles), or by digitonin treatment for removal of lysosomes [6] (filled circles). The efficiency of this treatment was indicated by a 95% loss of acid phosphatase activity in the mitochondrial extract. Squares refer to experiments with lysosome-depleted mitochondria in the absence (filled squares) or the presence of an extract from purified lysosomes corresponding to 310 μg protein/ml (open squares). The lysosomes (tritosomes) were prepared from livers of Triton WR 1339 pretreated rats according to [7]. Incubation was at 25°C.

it can be concluded that both the phosphorylated and the dephosphorylated forms of the enzyme were degraded at the same rate.

Further studies have shown that extracts from RLM and especially from rat liver lysosomes are capable in inactivating also highly purified PDH from pig heart muscle. On this basis we have developed now a standard assay system for the purification and further characterisation of the 'inactivase'. The phenomenon of PDH inactivation described in this communication resembles in many respects the inactivation of the 2-oxoglutarate dehydrogenase complex by extracts from bovine kidney mitochondria as reported by Linn [9]. Like in their experiments preliminary data from this laboratory have indicated that the three component enzymes of the PDH complex show no loss in activity when examined individually. This would mean that the 'inactivase' acts in some way by limited proteolysis leading to disaggregation of the multienzyme complex rather than by affecting the catalytic function of the single components.

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References

[1] Portenhauser, R. and Wieland, O. (1972) Eur. J. Biochem. 31, 308.
[2] Wieland, O. H., Löffler, G., Patzelt, C. and Portenhauser, R. (1974) Regulation of Hepatic Metabolism, p. 62. Munksgaard, Copenhagen.
[3] Tabor, H., Mehler, A. H. and Stadtman, E. R. (1953) J. Biol. Chem. 204, 127.

Table 1

Incubation (25°C) min	PDH-activity Before activation (= PDH_a)	After activation (= PDH_{total})	PDH_a : PDH_{total}
5	0.12	0.215	0.56
20	0.085	0.150	0.57
40	0.050	0.085	0.59

Extract prepared from non-preactivated RLM was incubated and at the times indicated samples were removed for PDH-determination either immediately (PDH_a) or after activation (total PDH activity). For activation 200 μl of extract were mixed with 25 μl 0.1 M $MgCl_2$, 40 μl RBS diluted 1:40 with 5 mM potassium phosphate buffer pH 7.0, 15 μl (approx. 20 μg protein) of purified heart muscle phosphatase [8], and incubated for 20 min at 25°C.

[4] Weiss, L., Löffler, G. and Wieland, O. H. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 363.
[5] Weiss, L. (1975) Habilitationsschrift Universität München.
[6] Loewenstein, J., Scholte, H. R. and Wit-Peeters, E. M. (1970) Biochim. Biophys. Acta 223, 432.
[7] Wattiaux, R., Wibo, M. and Baudhuin, P. (1962) Ciba Found. Symp. Lysosomes (Churchill, London) p. 176.
[8] Siess, E. A. and Wieland, O. H. (1972) Eur. J. Biochem. 26, 96.
[9] Linn, T. C. (1974) Arch. Biochem. Biophys. 161, 505.
[10] Gruder, W. G. and Wieland, O. H. (1974) Eur. J. Biochem. 42, 529.