A FACTOR ISOLATED FROM LIVER INHIBITS REASSOCIATION/REACTIVATION OF DISSOCIATED GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

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1. Introduction

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis, is composed of 4 identical subunits. This tetrameric structure is required for catalytic activity. Low pH and strong denaturants lead to dissociation into monomeric units and loss of enzymatic activity [1–3]. Removal of the denaturing conditions and separation of incorrectly folded aggregates lead to complete recovery of the specific enzymatic activity as well as the hydrodynamic and spectral properties [4,5]. NAD^+ enhances the rate of reactivation [1,2,6]. The purposes of this communication is to give evidence of a low molecular thermolabile factor, isolated from human liver which inhibits the reactivation in vitro of dissociated glyceraldehyde 3-phosphate dehydrogenase but has no direct influence on the catalytic activity of the tetramer.

2. Experimental

2.1. *Reassociation/reactivation assay*

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Boehringer) was prepared by repeated dialysis at 4°C against 0.01 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA and 5 mM dithioerythritol (DTE). Dissociation occurred after mixing 0.1 ml 0.01 M sodium phosphate buffer (pH 7.5) containing 10 μg GAPDH, 1 mM EDTA and 5 mM DTE, with 0.1 ml 0.1 M glycine/H_3PO_4 buffer (pH 2.5) which also contained 1 mM EDTA and 5 mM DTE, for 10 min at 22°C (final pH 2.8). The material with inhibitory properties was dissolved in 0.25 M sodium phosphate buffer (pH 7.64) and reactivation (22°C) was induced by adding 0.8 ml of this mixture to the dissociated enzyme. The pH was thereby adjusted to 7.5. The effect on the reactivation rate was followed by measuring the GAPDH-activity according to [3]. The final reaction mixture for activity measurement contained 0.23 μg GAPDH/ml, 1 mM D,L-glyceraldehyde 3-phosphate, 1 mM NAD^+, 0.01 M Na_2HAsO_4, 1 mM EDTA and 5 mM DTE in 0.05 M sodium diphosphate buffer (pH 8.5).

2.2. *Isolation of the inhibitor*

(i) Saline perfused deep-frozen liver slices (from human organ donors fatally injured in traffic accidents) (200 g) were thawed at 4°C, cut into small pieces and homogenized in 400 ml 0.5 M NaCl with a Waring homogenizer. The pH was kept at 2.0 by adding HCl. After centrifugation for 60 min at 2000 × *g* the floating lipid layer and the sediment were discarded. The remaining supernatant was brought to a final concentration of 6% trichloroacetic acid and centrifuged for 30 min at 2000 × *g*. The sediment was discarded and the supernatant extracted with diethyl ether to remove trichloroacetic acid. After ultrafiltration of the supernatant through an UM 10 Amicon membrane at pH 2.0, desalting with an UM 05 Amicon membrane at pH 8.0 and lyophilization, 1.2 g crude extract (M_r 500–10 000) were obtained.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); NAD^+, β-nicotinamide-adenine-dinucleotide, oxidized form; LDH, lactate dehydrogenase (EC 1.1.1.27); EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol

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(ii) This material was dissolved in 5 ml 0.43 M formic acid (pH 2.0) and chromatographed on a Sephadex G-25 column (100 × 2.5 cm, flow rate 14 ml/h, solvent 0.43 M HCOOH). The excluded volume was lyophilized and 105 mg of a white fluffy powder was obtained. It contained the inhibitory capacity. This material was devoid of the peptides reported [7] to inhibit the reactivation of LDH isoenzymes.

(iii) This material (102 mg) was dissolved in 6 ml 0.12 M CH_3COOH (pH 3.0) and ultracentrifuged for 30 min at 100 000 × *g*. The supernatant which was devoid of the inhibitor was discarded. The sediment was dissolved in 0.12 M CH_3COOH and lyophilized, yielding 22.5 mg white powder containing the material which inhibited the reactivation of GAPDH.

(iv) This material (16 mg) was chromatographed on a Sephadex G-50 column equilibrated with 0.25 M sodium phosphate buffer (pH 7.64). The elution profile is shown in fig.1.

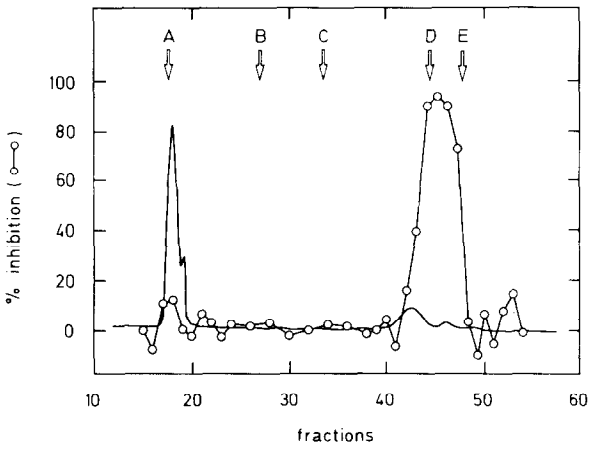

Fig.1. Chromatography on a Sephadex G-50 column in 0.25 M sodium phosphate buffer (pH 7.64). Column: 85 × 1.5 cm, elution rate 14 ml/h with fractions of 3.5 ml. Calibration was with: (A) albumin (exclusion volume); (B) cytochrome *c* (M_r 12 500); (C) corticotropin A (M_r 5300); (D) bacitracin (M_r 1460); (E) Thr–Lys–Pro–Arg (M_r 500). Aliquots of the effluent were withdrawn and tested for inhibition of GAPDH-reactivation. The effect of the inhibitor is expressed in % recovery of catalytic activity after 18 h reactivation, compared to a dissociated and reactivated control (○——○). The solid line represents the track of the UV-detector at 206 nm.

(v) The three most active fractions exhibiting ~90% inhibition (fig.1) were desalted over a Sephadex G-15 column using distilled water. After lyophilization 100 μg of a white powder was obtained (table 1).

3. Results and discussion

3.1. *Effect of the inhibitor on glyceraldehyde 3-phosphate dehydrogenase*

Native tetramers as well as reassociating subunits were used as targets of the inhibitor. The inhibitor had no immediate effect on the catalytic activity of the tetramer. An immediate effect of the inhibitor was found when it was exposed to dissociated GAPDH before reactivation. This strongly suggests that the inhibitor interferes in the folding/association/activation of inactive monomers to catalytically active tetramers. By using material obtained after the acid precipitation and ultracentrifugation a hyperbolic dose–response curve is obtained (fig.2). A concentration of 200 μg/ml was necessary to achieve half-maximal inhibition of the reactivation. By the last isolation step on Sephadex G-50 in 0.25 M sodium phosphate buffer (pH 7.64) purification of ~100-fold was achieved (fig.1). However the desalting procedure by Sephadex G-15 in distilled water resulted in a considerable loss of specific activity (table 1).

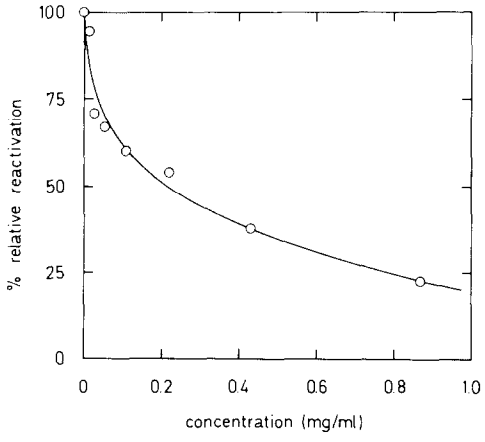

Fig.2. Dose–response curve. Inhibitor containing material obtained after precipitation with 0.12 M acetic acid was tested as described. Reactivation rate was determined after 18 h and compared with a control without inhibitor.

3.2. *Summary of the increase of the specific activity during the isolation described in section 2.2*

Table 1

Isolation step	Yield (mg)	Specific activity (conc. for 50% inhibition)
Starting material	200 000	–
(i) Crude extract	1200	Trichloroacetic acid contaminated
(ii) Sephadex G-25 column in 0.43 M HCOOH	105	900 μg/ml
(iii) Acid precipitation	22.5	210 μg/ml
(iv) Sephadex G-50 column in 0.25 M phosphate buffer (pH 7.64)	~0.1	~2 μg/ml
(v) Desalting (Sephadex G-15)	0.1	200 μg/ml

3.3. *Characterization of the inhibitor*

As shown in fig.1 the inhibitor emerges from the Sephadex G-50 column in 0.25 M sodium phosphate buffer (pH 7.64) as a symmetrical peak which corresponds to ~1000–1500 app. M_r. However, when chromatography was performed in 0.43 M HCOOH using Bio-Gel P-2, Sephadex G-25 or Sephadex G-50 columns most of the inhibitor emerges in the excluded volume. This apparent aggregation at low pH was used to purify the inhibitor by Sephadex G-25 and ultracentrifugation at pH 2–3.

Free-flow electrophoresis revealed a negative net charge of the inhibitor at pH 6.5. During all purification procedures performed the GAPDH-reactive factor was associated with ninhydrin-positive material.

A slow deactivation occurs at room temperature as well as in the cold when the inhibitor was stored in 0.25 M sodium phosphate buffer (pH 7.64). Moderate heating leads to rapid deactivation of the inhibitor (fig.3). Oxidation of essential SH-groups of GAPDH would also lead to a decreased recovery of enzymatic activity [2,5]. To exclude the possibility that the reduced recovery of catalytic activity was due to oxidation by an unspecific SH-group reagent, the inhibitor was incubated for 3 h at 22°C with 5 mM DTE. The properties of the DTE-treated inhibitor were identical to a control sample which was not DTE-treated.

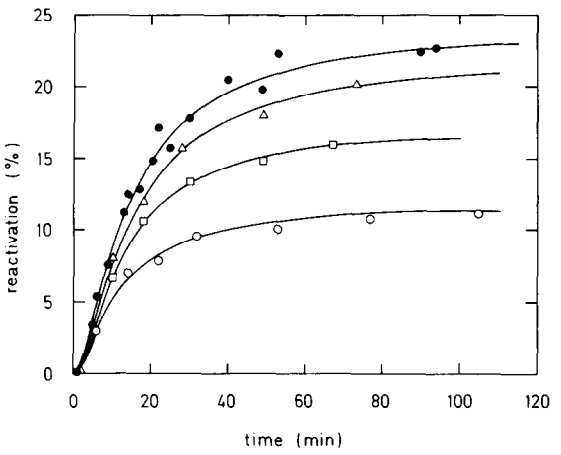

Fig.3. Time course of reactivation and effect of exposing the inhibitor to high temperature. Recovery of GAPDH-activity is expressed in % compared to fully associated enzyme: (•) control without inhibitor; (○) with 240 μg/ml of inhibitor-containing material; (□) same experiment but inhibitor exposed for 5 min to 60°C prior to the experiment; (△) inhibitor exposed for 5 min to 70°C.

4. Conclusions

In [7], two isoenzyme specific inhibitors of muscle-type and heart-type lactate dehydrogenase were shown. Both inhibitors were peptides with low M_r-values. They had no effect on the catalytic activity of the tetrameric isoenzymes but inhibited the reactivation of dissociated subunits. Here, we suggest that factors which affect the folding/association/activation of oligomeric enzymes might not be restricted to LDH isoenzymes but could represent a part of a general mechanism in metabolic regulation.

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