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ISOLATION OF A MITOCHONDRIAL FACTOR FROM RAT LIVER WHICH POTENTIATES THE INACTIVATION OF GLUTAMATE DEHYDROGENASE BY LYSOSOMES

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SUMMARY: Rat liver glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase, deaminating) E.C. 1.4.1.3.) is inactivated by the mitochondrial matrix in combination with lysosomal preparations. Neither lysosomal or mitochondrial matrix extracts per se inactivate the enzyme appreciably under the conditions used. Fractionation of the matrix indicates that a low molecular weight factor is responsible for the potentiation of inactivation of glutamate dehydrogenase by lysosomes. Its absorption spectrum and chromatographic behaviour suggest that this factor is NADP.

It is generally accepted that proteolysis and its regulation are important for the maintenance of steady state levels of intracellular proteins. However, the mechanisms are not clear. There is evidence of lysosomal participation, but other systems and factors seem to be involved (1). Modulation of proteins by covalent modifications, interactions with metabolites or allosteric effectors may change the susceptibility of proteins to proteases. Also, recently, the low molecular weight proteins, ubiquitin and a glycoprotein activator have been reported to initiate degradation of the substrate, protein or sphingolipid, to which they bind (2,3).

We have shown that rat liver carbamyl phosphate synthase and adenosine triphosphatase (ATPase) were inactivated at or near intracellular pH by component(s) of the inner mitochondrial membrane in combination with lysosomal enzymes (4). We

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selected next glutamate dehydrogenase because, like carbamyl phosphate synthase and ATPase, it is a mitochondrial enzyme and has a similar molecular weight but a different turnover (5). Now we show the presence in rat liver mitochondrial matrix of a low molecular weight factor, tentatively identified as NADP, that potentiates the inactivation of glutamate dehydrogenase by lysosomal extracts.

MATERIALS AND METHODS

Male Wistar rats between 200 and 300 g were used for all preparations of liver fractions, lysosomes and glutamate dehydrogenase. NADH, α-ketoglutaric acid, NADP and NADPH were from Boehringer-Mannheim; Diaflow Ultrafilters PM-10 and UM-2 from Amicon, PD-10 prepacked Sephadex G-25 M columns from Pharmacia Fine Chemicals. All other chemicals were reagent grade, from E. Merck.

Preparation of Mitoplasts. Rat liver mitochondria were prepared as described by Kun et al. (6). The mitochondria obtained were then used to prepare fractions thereof as previously described (4).

Fractionation of matrix. The soluble matrix fraction was separated into high and low molecular weight (∿5,000) fractions on a PD-10 Sephadex G-25 M column. Low molecular weight components were further purified and concentrated by ultrafiltration with a UM-2 membrane. From 10 to 200 µl of the fraction retained by the UM-2 membrane were chromatographed on acid washed paper 12 x 26 cm^2 using n-butanol : acetic acid : water, 60:20:20 (7) and descending chromatography for 5-6 hours. NADPH and NADP were used as markers. Location of spots was done with a UVSL-58 Mineral Light and ninhydrin (0.2% in acetone). Once detected, equal areas were cut, eluted with 50 mM potassium phosphate buffer, pH 6.0, 5 mM β-mercaptoethanol and assayed for inactivating activity. Other solvent systems used were isobutyric acid : concentrated NH_4OH : water, 63:1:33 and ethanol : 1.0 M ammonium acetate pH 7.5, 7:3 (8).

Preparation of lysosomes. Rat liver lysosomes were suspended in water and treated as described before (4). N-acetyl-β-D-glucosaminidase was assayed with the method of Findlay et al. (9).

Preparation of glutamate dehydrogenase. Glutamate dehydrogenase was isolated from rat liver according to Fahien et al. (10). It was kept in 28% ammonium sulfate and, when needed, desalted with Sephadex G-50 (11). It was assayed according to Grisolía et al. (12) with the following changes: in a total volume of 1.0 ml the assay mixture contained 43µmoles potassium phosphate buffer pH 7.6, 30µmoles α-ketoglutarate, 60 µmoles NH_4Cl, 0.25 µmoles NADH and the enzyme.

Inactivation assays. All incubations were carried out in 1.5 ml Eppendorf centrifuge tubes at pH 6.0 and 37°C for 30 min. At times indicated, 10 or 25 µl portions were mixed with 200 or 500 µl of cold 10 mM potassium phosphate buffer pH 7.4, 5 mM MSH, and portions of this mixture were assayed for glutamate dehydrogenase activity (12).

Protein determination. Proteins were determined according to Lowry et al. (13) or for mitochondria, mitoplasts and lysosomes with the biuret-deoxycholate assay according to Jacobs et al. (14).

RESULTS AND DISCUSSION

Lysosomes are potentially able to degrade almost any protein. It has been shown that mitoplast proteins are a good substrate with lysosome extracts (15). Because lysosomes are less active at neutral pH, we have carried out most of our studies at pH 6, at which glutamate dehydrogenase is reasonably stable under the conditions used in our experiments (Fig. 1). This figure also shows that glutamate dehydrogenase is stable to lysosomes and mitoplasts separately but is considerably less stable to lysosomes and mitoplasts combined. Mitoplasts without added lysosomes also produced some inactivation probably due to lysosomal contamination (see below).

In previous work we showed inactivation of carbamyl phosphate synthase and ATPase by mitochondrial inner membranes in combination with lysosomes (4). As can be seen in Fig. 2, mitoplast membranes had no marked effect while inactivation was produced by matrix, particularly in the presence of lysosomes.

We then tried to isolate the fraction responsible for the potentiation of lysosomes by separating the high from the low molecular weight components by passing the matrix extract through a Sephadex G-25 M column (see Materials and Methods). Table I shows the effect of matrix fractions. The highest inactivation occurred with the low molecular weight components. The high molecular weight fraction also showed residual inac-

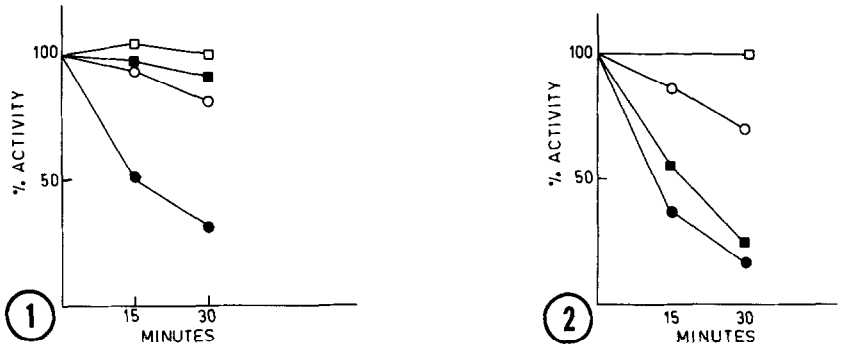


Fig. 1. Effects of mitoplasts and lysosomes on the activity of glutamate dehydrogenase. Incubation mixtures contained, per ml, 5 mg enzyme protein, 50 mg mitoplast protein and/or 2 mg lysosome protein containing 0.2 U of N-acetyl-β-D-glucosaminidase as shown. Incubations were at 37°C in 10 mM sodium citrate pH 6.0. At 0 time, 15 and 30 min, 25 µl aliquots were taken into 500 µl of cold 10 mM phosphate buffer pH 7.4 containing 5 mM β-mercaptoethanol, and assayed for enzyme activity as indicated in Methods. (□ - □) No Lysosomes added; (■ - ■) Lysosomes added; (O - O) Mitoplasts added; (● - ●) Lysosomes and mitoplasts added.

Fig. 2. Effect of mitoplast membranes, matrix and lysosomes on activity of glutamate dehydrogenase. Incubation mixtures contained, per ml, 5 mg enzyme protein, 25 mg matrix and/or 15 mg membrane protein and 2 mg lysosomal protein containing 0.2 U of N-acetyl-β-D-glucosaminidase, when indicated. Incubations were done in 10 mM sodium citrate pH 6.0 at 37°C. At 0 time, 15 and 30 min, 25 µl aliquots were taken into 500 µl cold 10 mM phosphate buffer pH 7.4 containing 5 mM β-mercaptoethanol and assayed for glutamate dehydrogenase activity, as indicated in Methods. (□ - □) Membranes and Lysosomes added; (O - O) Matrix added; (■ - ■) Matrix, Membranes and Lysosomes added; (● - ●) Matrix and Lysosomes added.

tivation activity, perhaps due to to insufficient separation of low molecular substances and proteins. Indeed, when we passed the matrix through Sephadex G-50 (11), the protein fraction had no inactivation effect. Thus, we concluded that the factor potentiating the inactivation of glutamate dehydrogenase by lysosomes was a compound of low molecular weight present in the mitochondrial matrix. Ultrafiltration with a PM-10 membrane located the activity in the filtrate, while a UM-2 membrane retained approximately 70% of the activity, indicating a molecular weight between 500 and 10,000.

Table I. Effect of mitochondrial matrix fractions and lysosomes on glutamate dehydrogenase.

Additions	Residual Activity %	
	Without Lysosomes	With Lysomomes
None	100	93
Matrix	73	16
Matrix: High Molecular Weight Fraction	73	40
Matrix: Low Molecular Weight Fraction	100	34
Matrix Desalted (11)	100	90

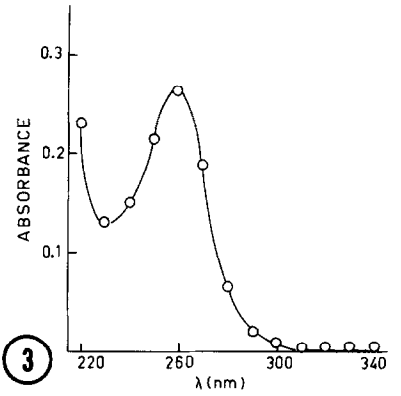
Incubation mixtures contained, per ml, 2.5 mg enzyme, 6 mg matrix proteins or equivalent amounts of matrix fractions, 0.15 U of N-acetyl-β-D-glucosaminidase in 90 mM potassium phosphate buffer pH 6.0 containing 4 mM β-mercaptoethanol for 30 minutes at 37°C.

The UM-2 retained fraction was chromatographed on paper, as described in Methods, using a butanol/acetic acid solution as solvent. We observed three main U.V. spots. The closest to the origin had the bulk of activity and it was ninhydrin negative.

The spectrum of the material eluted from this spot is shown in Fig. 3. The A250/A260 and A280/A260 ratios were 0.85 and 0.24, similar to those of pyridine nucleotide coenzymes (8).

When we chromatographed the factor, it had R_f values similar to NADP and NADPH using three solvent systems (see Methods).

As can be seen in Fig. 4, commercial preparations of NADP produced the same effect as the factor isolated from mitochondrial matrix. NADPH in the absence of lysosomes produced a considerable inactivation of glutamate dehydrogenase; this effect was also increased on addition of lysosomes.



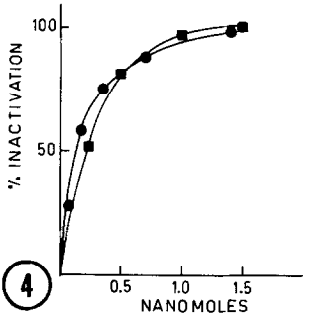


Fig. 3. Absorption spectrum of factor.

Fig. 4. Effect of different concentrations of factor and NADP on glutamate dehydrogenase activity. Incubations contained, per ml, in 90 mM potassium phosphate buffer pH 6.0 and 4 mM β-mercaptoethanol 2.5 mg enzyme protein, 7.5 mg bovine serum albumin, lysosomes containing .15 U of N-acetyl-β-D-glucosaminidase, and NADP or factor in the indicated amounts. Quantities of factor added were calculated from OD values at 260 nm, assuming the factor to be NADP. At 0 time and 30 min, 10 µl of incubation mixture were added to 200 µl of cold phosphate buffer and assayed as described in Methods. (■ - ■) NADP added; (● - ●) Factor added.

The above results suggest that the factor in the mitochondrial matrix which facilitates the inactivation of glutamate dehydrogenase by lysosomes is NADP. The total NADP(H) content in mitochondria is believed to be from 2-5 nmoles/mg protein (16) and the NADPH : NADP ratio 4:1 (17). Unfortunately, there do not appear to be data available for mitoplasts. Since we find ∿0.5 nmoles/mg protein and since the factor per se does not inactivate glutamate dehydrogenase, this would indicate that during the preparation of mitoplasts there is oxidation of NADPH to NADP, perhaps by NADPH oxidase, or else that the proportion of NADP in mitoplasts is higher than that reported in mitochondria. Since no NADPH was isolated and our total recovery of pyridine nucleotides is low, perhaps there is leakage and/or the bulk of NADPH has been converted into NADP. At any rate, since both NADP and NADPH

potentiate the inactivation of glutamate dehydrogenase by lysosomes, it seems that cofactors change the stability of enzymes as has long been suggested (18) but demonstrated here for the first time 'in vitro' with components isolated from the same source. In this regard, it is also important to point out that degradation of glutamine synthetase 'in vivo' is accelerated by glutamine and prevented by cell enucleation (19), indicating the existence of signals between synthesis and degradation of proteins.

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