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PROTEOLYTIC MODIFICATION OF RAT LIVER FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE BY ADMINISTRATION OF LEUPEPTIN IN VIVO

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Summary

When leupeptin, a thiol protease inhibitor of microbial origin, was injected into rats, the activity of fructose-1,6-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) in the liver decreased to about 60% of that in control rats. However, the concentration of aldolase protein in the liver extracts, measured with a specific antibody obtained with enzyme purified on a phosphocellulose column, remained unchanged.

Injection of leupeptin also caused a marked increase in the activities of free lysosomal proteases, such as cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.-), cathepsin D (EC 3.4.23.5) and lysosomal carboxypeptidase A in the cytosol fraction. A clear inverse relationship between aldolase and cathepsin B activities in the cytosol fraction was demonstrated. The possibility that the less active form of aldolase detected in the livers of leupeptin-treated rats was produced during homogenization was excluded by showing that the aldolase activity was not changed by addition of various protease inhibitors to the homogenization medium. When insulin was coinjected with leupeptin, increase in the activity of free cathepsin L and decrease of activity of aldolase produced by the injection of leupeptin was prevented. These findings indicate that modification of aldolase may be due to the action of a lysosomal protease(s). Enhanced sensitivity of lysosomes to osmotic shock was demonstrated in the livers of leupeptin-treated rats, suggesting that the lysosomal membrane is labilized by administration of leupeptin. Incubation of the purified aldolase with the lyso-

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; Cbz, carbobenzoxy; E-64, (*N*-(*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl)agmatine); Z-Glu-Phe, carbobenzoxy-L-glutamyl-L-phenylalanine.

somal fraction produced the same changes in properties of aldolase as those observed in vivo on injection of leupeptin.

Introduction

Previously we reported that administration of a microbial protease inhibitor, leupeptin or E-64 (*N*-(*N*-(L-3-*trans*carboxyoxirane-2-carbonyl)-L-leucyl)-agmatine), inhibited the activities of cathepsin B and cathepsin L in the lysosomal fraction of the liver and that these inhibitors also reduced the turnover rate of liver aldolase [1]. During these studies, we found that the activity of aldolase is consistently low in rats treated with protease inhibitors.

Therefore, in the present work we have examined the catalytic and molecular properties of aldolase from control and leupeptin-treated animals and found that enzyme purified from inhibitor-treated animals had low specific activity, although it showed no appreciable change in molecular weight. A possible explanation of these changes in properties of aldolase is proteolytic modification of the enzyme by lysosomal protease(s). We found that injection of leupeptin resulted in an increase in activity of free lysosomal proteases in the cytosol fraction and we also observed an inverse relationship between the activities of aldolase and cathepsin B, a lysosomal marker.

This paper reports the effects of administration of leupeptin in vivo on liver aldolase and reports evidence that a lysosomal protease(s) is involved in modification of the enzyme.

Materials and Methods

Materials. *N*-Benzoyl-DL-arginine-*p*-nitroanilide and azocasein were purchased from Sigma Chemical Co. Triose phosphate isomerase, glycerol-3-phosphate dehydrogenase and NADH (sodium salt) were from Boehringer, Mannheim, F.R.G. Z-Glu-Phe was from the Peptide Center (Institute for Protein Research, Osaka). Hemoglobin and *p*-phenyl phosphate were from Wako Pure Chemical Industry Co., Kyoto. Leupeptin and E-64 were kindly supplied by Dr. Aoyagi (Institute of Microbial Chemistry, Tokyo) and by Dr. Sawada (Taisho Pharmaceutical Co., Tokyo), respectively. DEAE-cellulose was from Whatman and phosphocellulose was from Serva, F.R.G. Regular insulin and triamcinolone acetonide were obtained from Shimizu Pharmaceutical Co., Japan and Sankyo Co., Japan, respectively. All other reagents were standard analytical grade products. Aldolase was purified from rat liver by the method of Rutter et al. [2], except that material was fractionated with 45—70%, not 45—60% $(NH_4)_2SO_4$.

Injection of protease inhibitor and preparation of the cytosol and lysosomal fractions. Leupeptin was injected subcutaneously as a 1 : 1 emulsion with complete adjuvant (DIFCO) at the doses indicated in the legends to figures and tables. Control rats were injected with oil only. The livers were removed from rats immediately after injection and weighed and chilled on ice. Samples of 5 g liver were minced and homogenized in 4 vol. cold 0.25 M sucrose, pH 7.0. The homogenate was centrifuged in 10 ml portions for 60 min at 105 000 × *g* and

the resulting supernatant was used as the cytosol fraction. The remaining homogenate was used to obtain the heavy particular fraction as described previously [1], and this is referred to here as the lysosomal fraction. The resulting lysosomal fraction was suspended in 5.8 ml of 0.25 M sucrose/ 1 mM EDTA, pH 7.0, and solubilized by addition of 0.2 ml 10% Triton X-100. This extract was used for assays of lysosomal enzymes.

Injection of insulin and triamcinolone. Rats were given 1.0 unit regular insulin intraperitoneally at zero time, followed by 1 unit at 3 h. Animals also received 2 ml 10% glucose at 0 and 3 h to prevent hypoglycemic shock. Triamcinolone acetonide (1 mg/100 g body weight) was injected intraperitoneally at zero time.

Enzyme assay. Fructose-bisphosphate aldolase was measured at 25°C by the method of Rutter et al. [2]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), malic enzyme (EC 1.1.1.40), phospho*enol*pyruvate carboxykinase (EC 4.1.1.32), pyruvate kinase (EC 2.7.1.40), malate dehydrogenase (EC 1.1.1.37) and lactic dehydrogenase were assayed by measuring the decrease or the increase in absorbance at 340 nm in a final volume of 1 ml at 25°C. Enzyme assays were performed by the methods of Rudack et al. [3], Hsu and Lardy [4], Tanaka et al. [5], Englard [6] and Stolzenbach [7], respectively. Cathepsin B was measured by the method of Otto and Bhakdi [8] with benzoyl-DL-arginine *p*-nitroanilide as a substrate. The *p*-nitroaniline liberated was converted to an azo-dye by diazotization and coupling, and its concentration was measured at 560 nm [9]. Cathepsin L and cathepsin D were determined by the methods of Kirschke et al. [10] and Matsuda and Misaka [11], respectively. In assay of cathepsin D, 20 μg/ml pepstatin were included in the control incubation mixture to measure the actual cathepsin D activity. Similarly, assay of cathepsin L was performed in the presence and absence of 40 μg/ml E-64, and cathepsin L activity was calculated as E-64-sensitive azocasein hydrolytic activity. 1 unit of activity is expressed as the amount of enzyme causing a change of 1 unit of absorption at A_{366}/min per ml. Lysosomal carboxypeptidase A activity was assayed with Z-Glu-Phe as substrate, by measuring the amount of ninhydrin-positive material in the trichloroacetic acid-soluble supernatant. The reaction mixture (1 ml) contained 0.05 M acetate buffer, pH 5.5/5 mM Z-Glu-Phe and an appropriate amount of enzyme protein. Incubation was carried out for 30—120 min at 37°C. Acid phosphatase (EC 3.1.3.2) was measured as described by Igarashi and Hollander [12].

Protein determination. Protein was measured by the method of Lowry et al. [13] with crystalline bovine serum albumin as a standard.

Immunological techniques. Anti-aldolase antiserum was raised in a goat immunized with homogeneous rat liver aldolase prepared as described above. The antiserum was monospecific, when analysed by Ouchterlony immunodiffusion and immunoelectrophoresis. Immunotitration was performed on the 105 000 × *g* supernatant solution obtained from the livers of control and leupeptin-treated rats.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [14] with 10% or 12% gel. Gels were fixed and stained with 7% acetic acid/25% methanol/0.1% Coomassie brilliant blue (v/v/w) for 1 h and destained with 7% acetic acid/25% methanol (v/v).

Assessment of lysosomal osmotic sensitivity. Rats were injected with 0.8 mg leupeptin/100 g body weight. The rats were killed 6 h after the injection and their liver was removed. Three livers were combined and chopped up with scissors, and 2.0 g minced liver was homogenized in a Potter-Elvejhem homogenizer with 4 vol. of 1 mM EDTA, pH 7.0, in water or sucrose solution at the concentration indicated in the figures. The homogenate was centrifuged at 105 000 × *g* for 60 min and the supernatants obtained were used for assay of acid phosphatase and lysosomal carboxypeptidase A.

Results

Decrease of rat liver aldolase activity on administration of leupeptin in vivo

Leupeptin was injected subcutaneously as a 1 : 1 oil emulsion into unstarved rats at a dose of 1.0 mg/rat, and the cytosol fractions from the liver of control rats and rats, 6 h after the injection of leupeptin, were assayed of activities of various cytosolic enzymes. The results in Table I show that administration of leupeptin caused a decrease in aldolase activity to about 60% of the control value and a slight increase in glucose-6-phosphate dehydrogenase activity, but it did not significantly affect the levels of other enzymes. For examination of the properties of aldolase, the enzyme from the liver of control rats and rats treated with protease inhibitor was purified by chromatography on phosphocellulose. Recoveries of two types of enzymes from the column were more than 80% and there was not much difference between them. The decrease of aldolase activity in the crude extracts on injection of leupeptin was associated with decrease in the specific activity of the purified enzyme (Table II). The ratios of the specific activities of the crude and purified enzyme preparations were similar in control and leupeptin-treated rats, indicating that after injection of leupeptin rat liver contains a less active form of aldolase with a lower specific activity. Accumulation of this less active aldolase was also demonstrated by titration of crude extracts with anti-aldolase antibody. Nearly 1,5-times more antibody was required to precipitate the same amount of enzyme activity in extracts of liver from leupeptin-treated rats. However, no change in

TABLE I

ACTIVITY OF VARIOUS CYTOSOLIC ENZYMES IN LIVERS FROM CONTROL AND LEUPEPTIN-TREATED RATS

Rats weighing 120–140 g were injected subcutaneously with 0.8 mg/100 g body weight of leupeptin as an oil emulsion. Control rats were injected with only oil. Rats were killed at 6 h after the injection and the cytosol fraction from the liver was used for assay of various enzymes. Values represent means of activity of five rats ± S.D. (μmol/min per g liver).

Treatment	Aldolase	Glucose-6-phosphate dehydrogenase	Malic enzyme	Phospho*enol*-pyruvate carboxykinase	Pyruvate kinase	Malate dehydrogenase	Lactic dehydrogenase
Control	8.4 ± 0.5	2.6 ± 0.5	2.8 ± 0.3	3.4 ± 0.3	3.6 ± 0.6	154 ± 4	229 ± 20
Leupeptin-treated	5.2 ± 0.4	3.4 ± 0.1	2.9 ± 0.4	3.9 ± 0.4	4.1 ± 0.6	179 ± 14	230 ± 25

TABLE II

PROPERTIES OF ALDOLASE FROM LIVERS OF CONTROL AND LEUPEPTIN-TREATED RATS

Leupeptin was injected subcutaneously at a dose of 0.8 mg/100 g body weight as an oil emulsion. Control rats were injected with only oil. Rats were killed at 6 h after the injection and the cytosol fraction was obtained. Aldolase was purified on a phosphocellulose column from the livers of five rats. Immunotitration of aldolase in liver cytosol fractions from control and leupeptin-treated rats was performed as follows: to 0.1 unit aldolase different amounts of antiserum were added in a final volume of 1.0 ml. The mixtures were then incubated at 25°C for 30 min and kept overnight at 4°C and the centrifuged supernatants (3000 rev./min for 10 min) were used for assay of aldolase activity.

Treatment	Aldolase activity		Immunotitration (ml antibody/unit aldolase)
	Cytosol fractions (units/mg protein)	Purified enzyme (units/mg protein)	
Control	0.140	4.27	0.24
Leupeptin-treated	0.099	3.02	0.34

the apparent molecular weight of the enzyme was detected by SDS-polyacrylamide gel electrophoresis (data not shown).

Increase of activities of lysosomal enzymes in the cytosol after administration of leupeptin in vivo

There are two possible explanations for the accumulation of the less active form of aldolase: covalent modification or limited proteolysis of the enzyme. It is known that addition of chloroquine to lysosomes in vitro causes inhibition of lysosomal cathepsin B [15] and release of the enzyme(s) into the medium [16]. Previously [1] we found that administration of leupeptin or E-64 to rats inhibited the lysosomal cathepsin B and cathepsin L activity in their liver. Therefore, in this work we examined the second possibility by measuring the levels of lysosomal enzymes in the cytosol fractions of the liver of control and leupeptin-treated rats. Leupeptin was injected subcutaneously. The rats were killed 6 h after the injection and the lysosomal and cytosol fractions of their liver were separated. As shown in Table III, leupeptin treatment resulted in marked inhibition of cathepsin B and cathepsin L in the lysosomal fraction, as described previously [1], and decrease in the total activities of the two cathepsins. Cathepsin B, L and D are normally associated with lysosomes, but the distribution of these lysosomal markers was altered by treatment with leupeptin, the activity of the free enzyme in the cytosol fraction increasing markedly. Another lysosomal marker, acid phosphatase, also showed a similar change in subcellular distribution after leupeptin treatment, although the activity of the free enzyme in the cytosol fraction of the livers were already high in control rats.

A significant relation between increase of free lysosomal proteases and decrease of aldolase was demonstrated by plotting the values for these two activities, found in the liver of rats injected with various concentrations of leupeptin. As shown in Fig. 1, a clear inverse relationship between free cathepsin B activity and aldolase activity was observed, suggesting that free lysosomal enzyme(s) released from the lysosomes is involved in formation of the less active form of aldolase.

TABLE III

EFFECT OF ADMINISTRATION OF LEUPEPTIN ON THE LEVELS AND DISTRIBUTIONS OF LYSOSOMAL ENZYMES IN RAT LIVER

0.8 mg/100 g body weight of leupeptin was injected subcutaneously as an oil emulsion. Rats were killed 6 h after the injection and the cytosol lysosomal fractions were used for measurement of the activities of cathepsins (B, L and D) and acid phosphatase. Values are means ± S.D. for four rats.

Enzyme	Cell fraction	Control	Leupeptin-treated
Cathepsin B	free	0.25 ± 0.05	0.90 ± 0.12
(mU/g liver)	bound	6.90 ± 1.41	0.95 ± 0.20
	free + bound	7.1	1.85
Cathepsin L	free	2.72 ± 0.91	9.72 ± 2.80
(mU/g liver)	bound	31.0 ± 5.00	9.80 ± 2.40
	free + bound	33.7	19.5
Cathepsin D	free	0.043 ± 0.020	0.36 ± 0.10
(Leu mequiv./min	bound	0.67 ± 0.12	0.19 ± 0.04
per g liver)	free + bound	0.71	0.56
Acid-phosphatase	free	2.12 ± 0.31	3.88 ± 0.50
(U/g liver)	bound	3.38 ± 0.48	1.50 ± 0.28
	free + bound	5.50	5.38

Effects of addition of protease inhibitors during homogenization on the activities of aldolase and free lysosomal proteases

To examine the possibility that the less active form of aldolase is formed not in vivo, but by proteolytic modification during homogenization, we added protease inhibitors to the medium during homogenization. Homogenates were prepared in 0.25 M sucrose, pH 7.0/1 mM PMSF/40 μg/ml E-64/20 μg/ml pep-

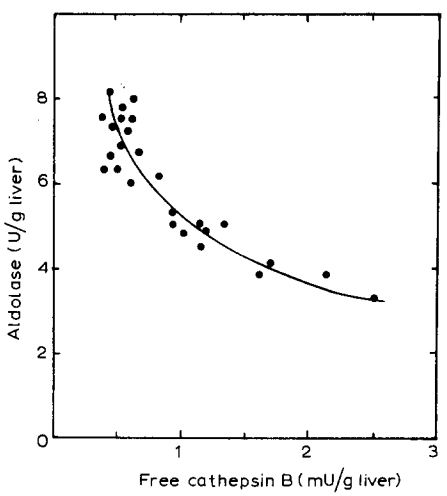

Fig. 1. Reciprocal relation between the activities of aldolase and cathepsin B in the cytosol fraction from the livers of rats treated with leupeptin. Rats weighing 140—170 g were injected subcutaneously 1—5-times with various amounts of leupeptin. Injections were given at 8 h intervals. The animals were killed 4 h after the final injection and the supernatants, obtained from their livers by centrifugation at 105 000 × *g* for 60 min, were used to measure the activities of fructose-bisphosphate aldolase and cathepsin B.

TABLE IV

CHANGES IN ALDOLASE AND LYSOSOMAL ENZYME ACTIVITY IN THE CYTOSOL FRACTION OF THE LIVER FROM CONTROL AND LEUPEPTIN-TREATED RATS MEASURED WITH AND WITHOUT PROTEASE INHIBITORS DURING HOMOGENIZATION

Doses of 1.0 mg leupeptin were injected subcutaneously as an emulsion with oil. Control rats were injected with oil only. Rats were killed 6 h after the injection. Crude extracts were prepared with a Potter-Elvehjem homogenizer using 2.0 g liver from control and leupeptin-treated rats and 4 ml 0.25 M sucrose, pH 7.0, or 0.25 M sucrose, pH 7.0/1 mM PMSF/20 μg/ml pepstatin/40 μg/ml E-64. The homogenates were centrifuged for 60 min at 105 000 × *g* and the supernatant solutions were used for assay of aldolase and lysosomal enzymes. Values are means ± S.D. for three rats.

Addition	Treatment	Aldolase (U/g liver)	Cathepsin B (mU/g liver)	Cathepsin L (mU/g liver)	Cathepsin D (Leu mequiv./ min/g liver)	Lysosomal carboxy-peptidase A (Phe mequiv./ min/g liver)	Acid-phosphatase (U/g liver)
None	Control	6.17 ± 0.19	0.388 ± 0.061	2.82 ± 0.28	0.080 ± 0.016	0.102 ± 0.016	2.10 ± 0.03
	Leupeptin-treated	4.78 ± 0.39	0.936 ± 0.151	10.9 ± 3.00	0.391 ± 0.058	1.11 ± 0.20	2.82 ± 0.19
Protease inhibitors	Control	6.00 ± 0.22	0.087 ± 0.044	1.66 ± 0.15	0	0.059 ± 0.014	2.18 ± 0.14
	Leupeptin-treated	4.69 ± 0.33	0.151 ± 0.023	2.14 ± 0.20	0	0.297 ± 0.171	2.64 ± 0.15

statin, and activities were measured in the supernatant obtained by centrifugation at 105 000 × *g* for 60 min. As shown in Table IV, similar increases of cathepsin B, L and D, to those shown in Table II and of lysosomal carboxypeptidase A were observed in leupeptin-treated rats. In the presence of protease inhibitors, the activities of the two thiol proteases (cathepsin B and cathepsin L) and cathepsin D in preparations from leupeptin-treated rats were reduced to less than the values of preparations from control rats. The activity of a serine protease, lysosomal carboxypeptidase A, was also reduced to 50—60% of that without protease inhibitors, but no significant inhibition of acid phosphatase was observed. Thus, aldolase activity decreased even under conditions when proteases that inactivate aldolase were inhibited. These results indicate that formation of a less active form of aldolase is due to the action of leupeptin in the liver, and is not an in vitro artifact.

Effect of insulin and triamcinolone on decrease of aldolase activity and increases of free activities of lysosomal proteases by leupeptin

Increased fragility of liver lysosomes has been reported following administration of glucagon to intact animals [17] or during perfusion of liver in situ [18]. Neely et al. [18] demonstrated that this induced change in lysosomes was prevented by the addition of insulin to the perfusion medium. Glucocorticoid is also known to have a membrane-stabilizing effect [19,20]. Then, the influence of these hormones on the increase of the activity of free lysosomal proteases and decrease of aldolase activity produced by the injection of leupeptin was tested. Rats were given insulin or triamcinolone with leupeptin simultaneously and killed 6 h after injection. As shown in Table V, leupeptin caused a several-fold increase of free cathepsin L activity and a decrease in aldolase activity in cytosol fractions of liver. Both effects of leupeptin were markedly prevented by the coinjection of insulin and slightly by the coinjection of triamcinolone. A single injection of insulin had no effect on the levels of free cathepsin L activity or aldolase activity within 6 h (data not shown). These results confirm a close relationship between the increase of free activity

TABLE V

EFFECT OF INSULIN AND TRIAMCINOLONE ON THE INCREASE OF ALDOLASE ACTIVITY AND FREE CATHEPSIN L ACTIVITY BY LEUPEPTIN

Leupeptin (0.8 mg/100 g body weight) was injected subcutaneously as an oil emulsion. Insulin (1.0 unit/100 g body weight) was given intraperitoneally at the same time as leupeptin and 3 h after the first injection. Rats also received 2 ml of 10% glucose at 0 and 3 h to prevent hypoglycenic shock. Triamcinolone (1 mg/100 g body weight) was injected intraperitoneally once at the same time as leupeptin. Animals were killed 6 h after injections and the cytosol fraction from the liver was used for assay of the activity of aldolase and cathepsin L. Values represent means of activities for four to five rats ± S.D.

Treatment	Aldolase activity (μmol/min per g liver)	Free cathepsin L activity (A_{366}/min per g liver × 10^{-3})
Control	8.36 ± 0.50	2.4 ± 0.2
Leupeptin	4.63 ± 0.35	15.0 ± 2.8
Leupeptin + insulin	7.00 ± 0.35	4.4 ± 1.5
Leupeptin + triamcinolone	5.27 ± 0.40	8.6 ± 1.3

of lysosomal proteases and decrease of aldolase activity, and could also suggest that administration of leupeptin increases the fragility of liver lysosomes.

Osmotic fragility of lysosomes prepared from the livers of control rats and rats treated with protease inhibitors

The osmotic fragility of lysosomes prepared from the livers of control rats and rats treated with leupeptin were next examined. When homogenates were prepared in hypotonic sucrose solution, acid phosphatase and lysosomal carboxypeptidase A activities were released into the soluble fraction (Fig. 2). Leupeptin shifted the curve for osmotic fragility to the left; that is, it increased the sensitivity of liver homogenates to osmotic shock. These results and the finding that the cytosolic activity of all lysosomal markers tested was increased by leupeptin administration, suggest that increases in the activities of free lysosomal enzymes on administration of leupeptin are due to non-selective release of enzymes from the lysosomes.

Inactivation of aldolase by the lysosomal fraction in vitro

The lysosomal fraction was prepared from the livers of untreated rats as described in Materials and Methods. The preparation was freeze-thawed 3-times

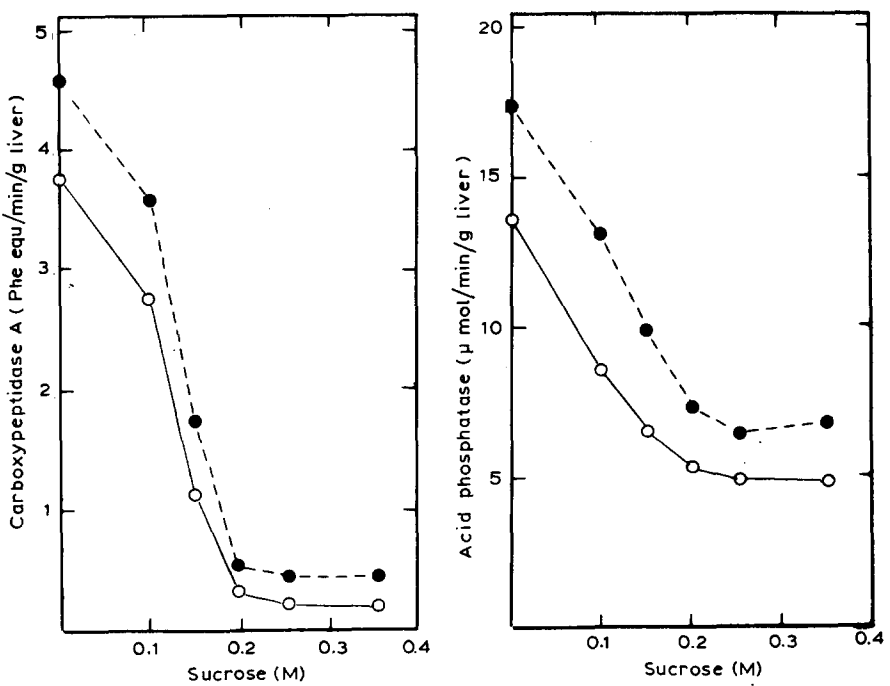

Fig. 2. Effect of extraction with hypotonic sucrose solutions on the cytosolic activities of lysosomal carboxypeptidase A and acid-phosphatase from control and leupeptin-treated rats. Rats weighing 150—170 g were injected subcutaneously with an oil emulsion 0.8 mg/100 g body weight of leupeptin or oil. They were killed 6 h after the injection and 3 g liver from control or leupeptin-treated rats were homogenized in 4 vol. water/1 mM EDTA, pH 7, or sucrose solutions of the concentrations indicated, also containing EDTA and adjusted to pH 7.0. The homogenates were centrifuged at 105 000 X *g* for 60 min and the supernatants were used to measure the activities of lysosomal carboxypeptidase A and acid phosphatase. (○———○), control rats; (●- - - - -●), leupeptin-treated rats.

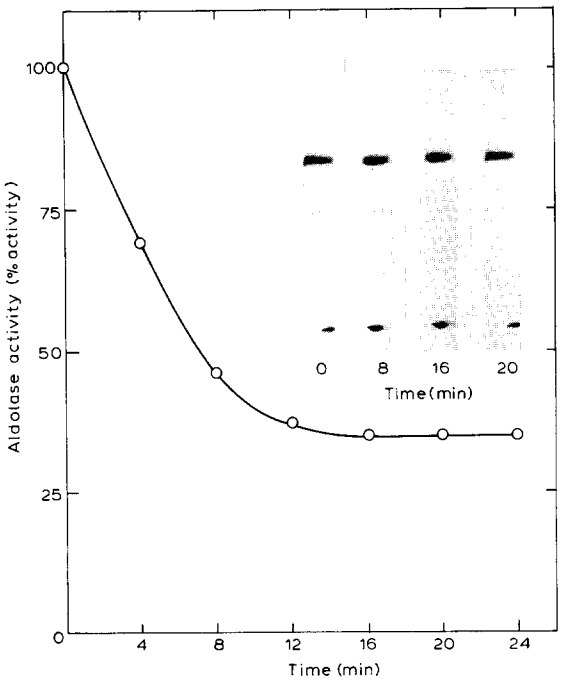

Fig. 3. Effect of released lysosomal enzyme(s) on native aldolase. Native fructose-bisphosphate aldolase was incubated at 25°C and pH 6.0 with the lysosomal fraction (2 mg/ml) prepared by repeated freeze-thawing. At the times indicated, the aldolase was purified from the reaction mixture by chromatography on phosphocellulose and its subunit composition was examined. 10 μg enzyme were applied to an SDS-polyacrylamide gel. The inset shows the electrophoretic patterns obtained at the times indicated.

and centrifuged at 11 000 × *g* for 20 min and the resulting supernatant was used for the following experiment. Purified aldolase was incubated at 25°C and pH 6.0 with the lysosomal preparation and at the times indicated in Fig. 3 samples were taken for assay of aldolase activity and for purification of aldolase, by chromatography on phosphocellulose. As seen in Fig. 3, despite progressive decrease of aldolase activity, no changes in the apparent molecular weight of subunits were detectable by SDS-polyacrylamide gel electrophoresis. Inactivation of aldolase by the lysosomes was less than 70%, and complete inactivation did not occur even on addition of a large amount of the lysosomal preparation. These observations indicate that the lysosomal preparation causes changes in the properties of aldolase similar to those observed on administration of leupeptin in vivo.

Discussion

The present results clearly show that on administration of the thiol protease inhibitor leupeptin, rat liver aldolase undergoes significant changes in catalytic and molecular properties, accompanied by functional changes in the lysosomes. There are many reports on the effects of leupeptin on the rates of synthesis and degradation of liver proteins [21—23]. When it is administered in vivo [1] or added to the culture medium of isolated liver cells [22,23], it has no effect on

the synthetic rate of hepatic proteins or on that of a selected enzyme, but significantly retards the degradation rate of liver enzymes or total proteins in liver cells. Injection of leupeptin into rats [1] actually inhibits the activities of cathepsin B and cathepsin L in the lysosomal fraction of the liver cells. However, there is a report of an unexpected effect of leupeptin: Tanaka et al. [24] demonstrated that addition of leupeptin and antipain to adult rat hepatocytes in primary culture caused induction of the acid protease, hemoglobin hydrolase. We found that injection of leupeptin caused an increase in the total activity of hemoglobin hydrolase (data not shown), but that it did not effect E-64-sensitive hemoglobin hydrolase, which is named cathepsin D in this paper (Table III). The effect of leupeptin in vivo shown in the present experiments, indicates that caution is required in the use of leupeptin in vivo.

The possibility that proteolytic modifications of native enzymes may occur after tissue homogenization, not in vivo, should be considered when studying enzymes in general. Petell and Libherz [25] studied the production of defective liver aldolase in aged mice, finding that the defective molecules are produced during storage of samples from liver of old rats rather than being produced in the liver of these rats in vivo. They also showed that inactivation of aldolase could be completely prevented by addition of PMSF and leupeptin during homogenization to inhibit proteolysis. However, we were not able to prevent inactivation of aldolase in the liver of leupeptin-treated rats by inclusion of PMSF and leupeptin during enzyme isolation (Table IV). Thus, we conclude that the inactive, or less active, form of aldolase observed in extracts of leupeptin-treated rat liver is produced by postsynthetic modification occurring in vivo.

The two types of aldolase molecules isolated from the livers of control and leupeptin-treated rats, respectively, were not distinguishable on the basis of their electrophoretic mobilities, molecular weights, or behavior on affinity chromatography, suggesting that specific limited proteolytic modification of aldolase occurs on administration of leupeptin. Properties of the less active form of aldolase are now being investigated in detail. Our preliminary experiments indicated that a decrease of aldolase activity in the liver of leupeptin-treated rats is attributable to hydrolysis of a peptide linkage(s) near the COOH-terminal of the polypeptide chain [26]. Aldolase has been shown to be inactivated preferentially by cathepsin B [27] and cathepsin L [28]. Nakai et al. [29] reported that treatment of pure rabbit muscle aldolase with cathepsin B resulted in inactivation of the enzyme activity with concomitant release of a dipeptide from the COOH-terminal of the molecule. But, no detectable change in subunit molecular weight could be detected after this treatment. In this work we observed increases in the cytosolic activity of cathepsin B and cathepsin L (Table III) on administration of leupeptin. In vitro experiments with the lysosomal extract, shown in Fig. 3, also support the idea that lysosomal proteases are involved in the modification of liver aldolase. The accompanying paper shows that lysosomal carboxypeptidase A and cathepsin B are involved in inactivation of aldolase in lysosomes.

Injection of leupeptin produced increases in osmotic fragility of lysosomes (Fig. 2), which could be due to an increase in lysosomal size [30] or due to the formation of autophagic vacuoles [31]. Leupeptin was recently found to cause

an increase in lysosomal size (Goldberg, A.L., personal communication).

Gluconeogenic stimuli such as starvation, diabetes and administration of glucocorticoid induce both the formation of autophagic vacuoles and the release of specific lysosomal proteases [32,33], and then result in proteolytic modification of fructose bisphosphatase [32] and in the decrease of aldolase activity [34]. Insulin antagonized the release of lysosomal protease(s) into the cytosol effected by either gluconeogenic stimuli [32] or by the injection of leupeptin (Table IV).

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