INSULIN DEGRADATION BY LYSOSOMAL EXTRACTS FROM RAT LIVER; MODEL FOR A ROLE OF LYSOSOMES IN HORMONE DEGRADATION

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SUMMARY: The presence of both glutathione-insulin transhydrogenase and "insulin specific protease" in highly purified lysosomal extracts from rat liver is presented. Although both of these enzyme activities were known to exist in the cytosol, it was difficult to understand their participation in insulin degradation, for it is known that insulin binds to plasma membrane receptors. Thus, the presence of both enzymes in lysosomes is of much interest for it suggests an explanation and/or model for the degradation via pinocytosis of insulin and possibly of other hormones which bind to receptors in the plasma membrane.

INTRODUCTION

Although the degradation of insulin in organs and tissues thereof has been extensively investigated during the last 25 years, we still do not know exactly where and how it occurs. It seems, however, that the most active organs are liver and kidney and that both glutathione-insulin transhydrogenase (1) (GIT, thiol: protein disulfide oxidoreductase, EC 1.8.4.2) present largely in the microsomal fraction of cells (2) and "insulin specific protease" found in the cytosol (3) participate in the process. Indeed, Varandani (4) has reported that in all rat tissues tested insulin is metabolized "in a sequential manner: first, the hormone is split at the disulfide bonds under the action of the glutathione-insulin transhydrogenase with the formation of A and B chains, and second, the resulting polypeptide chains are further catabolized by proteolysis".

While studying the degradation of proteins by lysosomes and lysosomal extracts, we noticed marked inhibition of the degradation of cytosol proteins of rat liver by insulin. We have found that extracts of purified lysosomes contain both glutathione-insulin transhydrogenase and protease activity.

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MATERIALS AND METHODS

L-[4,5-^{3}H]Leucine, 55 Ci/mmol, was from Amersham. Insulin, 4 mg/ml, was from Squibb; glutathione reductase from yeast (125 U/mg), NADPH and crystalline insulin from bovine pancreas were from Sigma Chemical Company; specially purified ninhydrin was purchased from Pierce Chemical Company; reduced glutathione was from Calbiochem, and Sephadex G-25 fine was from Pharmacia. All other chemicals were reagent grade.

Lysosomal extracts were prepared according to Ragab _et al._, (5) with the following changes: from 3 to 8 rats weighing 400-450 g were used for one preparation, the lysosomal pellet 2 was suspended in 3.0-5.0 ml of 0.2 M sodium citrate buffer, pH 5.0, frozen and thawed ten times, and then centrifuged in a Sorvall RC-2 centrifuge for 45 min at 48,000 x g; 5.0 ml of the supernatant were then passed through a 2 x 39 cm Sephadex G-25 fine column; 0.005 M KCl was used to equilibrate and to elute the column. The main fraction contained from 0.3-0.8 mg protein/ml. It was divided in small aliquots which were kept frozen at -20°C. It remained active for at least 3 weeks.

Labeled protein was prepared by injecting a 450 g rat i.p. with 375 µCi[^{3}H]Leucine (1000 µCi/µmole L-Leucine). After four hours, the rat was killed, the liver removed and a 1:3 isotonic sucrose homogenate prepared and fractionated according to the method of Ragab _et al._, (5). The microsome-free supernatant (100,000 x g, 60 min) was separated from small molecular weight radioactive components by filtration through a 2 x 39 cm Sephadex G-25 column, equilibrated and eluted with 0.005 M KCl. The main fraction contained from 3.2 to 6.3 mg protein/ml and 16,400 dpm/mg protein. It was divided in 5.0 ml aliquots which were kept frozen.

The procedure of Segal _et al._, (6) was adapted to determine the effect of lysosomal extracts on insulin. Incubation mixtures contained per ml approximately 1 mg [^{3}H]labeled protein, 0.1 mg of lysosomal protein, 0.1 mM sodium citrate, pH 5.0, and 0.02 mM mercaptoethanol. Insulin was added in varying amounts. The mixtures were incubated at 37°C. Routinely, 0.4 ml were taken at 5 and 45 min, mixed with 0.4 ml of 10% trichloroacetic acid and centrifuged. 0.5 ml aliquots of the supernatant were added to 10.0 ml of Aquasol in scintillation vials and radioactivity determined with a Nuclear Chicago Isocap/300 Liquid Scintillation System.

Amino acid release due to insulin degradation was also tested with ninhydrin (7). Mixtures containing insulin, lysosomal protein or both in 0.2 ml of 0.1 mM sodium citrate, pH 5.0, were incubated at 37°C. At appropriate intervals the mixtures were treated with 0.1 ml of 30% trichloroacetic acid. After 10 min at 0°C they were centrifuged. 0.2 ml of the supernatants were mixed with 0.1 ml of 1.5 M NaOH and 0.2 ml of 1.0 M sodium citrate, pH 5.0 and assayed (7). L-Leucine was used as a standard.

Glutathione-insulin transhydrogenase was assayed essentially according to Varandani and Tomizawa (8) with the following changes: the incubation mixture contained in 1.4 ml 0.07 mM NADPH, 0.14 mM GSH, 100 µg of glutathione reductase, 4 mg insulin, and varying amounts of the lysosomal extract.

RESULTS AND DISCUSSION

One mg of lysosomal protein degraded 1.65 mg of (radioactive) liver protein and, as judged by decrease of trichloroacetic acid soluble radioactivity by insulin, 0.99 mg (0.165 µmoles) of insulin in 40 min at 37°C (Fig 1). The average

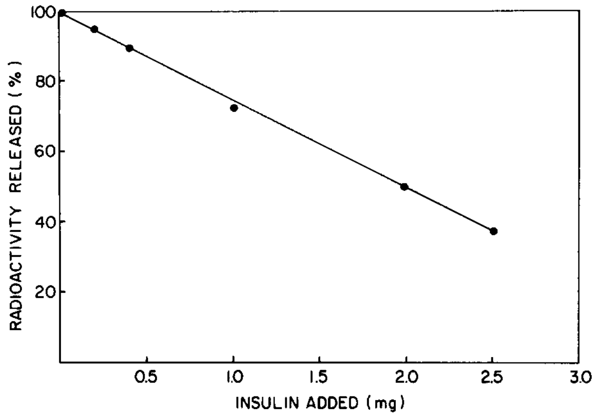

Fig 1. Release of radioactivity from labeled protein by lysosomal extracts. 1.0 ml incubation mixtures containing approximately 1.0 mg labeled protein, 0.1 mg lysosomal protein, 0.1 mM sodium citrate, pH 5.0, and 0.02 mM mercaptoethanol and the indicated amounts of insulin were incubated at 37°C for 40 min. Radioactivity was determined in the trichloroacetic acid supernatants.

yield in our preparations was 0.067 mg of lysosomal protein per g of liver. Therefore, the lysosomal extract corresponding to one g of liver will break down at least 1.6 μg insulin per min (this is a most conservative figure because lysosomal purity rather than yield was sought).

Insulin obtained from Squibb (containing 0.2% phenol as a preservative) inhibited the degradation of the labeled protein more than insulin without phenol. It was found experimentally that the additional inhibition was indeed due to the phenol.

Since inhibition of degradation of other proteins does not of itself indicate that insulin was degraded, we tested proteolysis directly by amino acid release. This is illustrated in Tables 1 and 2. Since glutathione-insulin transhydrogenase can use a variety of SH reagents (9), we tested the effect of glutathione and of mercaptoethanol. As shown in the Tables, both stimulated insulin degradation. This effect is most noticeable at higher levels of insulin. It should be noted that the Km for insulin with the transhydrogenase is a function of the type and concentration of thiols (9). At any rate, it can be calculated from the data of the tables that the addition of 1 mg of lysosomal protein

Table 1

Effect of SH reagents and lysosomal protein on the degradation of insulin

SH Reagent added	μg lysosomal protein used				
	2	5	15	30	120
	nanomoles aminoacid liberated/h				
None	8	20	62	120	470
MSH	10	33	105	190	620
GSH				200	680

The indicated amounts of lysosomal protein and 400 μg of insulin in 0.2 ml were incubated for 1 h (with 15 μg lysosomal protein the incubation was for 2 h and with 2 or 5 μg lysosomal protein for 4 h). When used, the incubation mixtures contained 0.2 mM mercaptoethanol (MSH) or 0.1 mM glutathione (GSH). The values obtained for the longer incubations have been normalized to 1 h.

Table 2

The effect of insulin levels and SH reagents on proteolysis by lysosomal protein

Insulin added	SH Reagent added		
	NONE	MSH	GSH
μg	nanomoles aminoacid liberated/h		
40	120	130	130
80	170	190	230
200	180	280	335

The indicated insulin and 60 μg lysosomal protein (120 μg when using 200 μg insulin) in 0.2 ml were incubated for 1 h. When used, the incubation mixtures contained 0.2 mM MSH or 0.1 mM GSH. (The values with 120 μg lysosomal protein have been normalized to 60 μg for easier comparison.)

to insulin would result in the release ∿5 μmoles of amino acid per h at 37°C, or, assuming an average molecular weight of ∿100 per amino acid and complete hydrolysis, 0.86 μg amino acid per g liver per min.

When lysosomal extracts were assayed for glutathione-insulin transhydrogenase they showed activity sufficient to reduce 0.36 μmoles of NADP or insulin per 1.0 mg of lysosomal protein in 60 min at 37°C, or 2.4 μg insulin per g of liver per min. This represents ∿1.5 times an excess to split the A and B chains of insulin over the capacity to hydrolyze A and B chains by the protease present in the lysosomal extracts.

These results are of interest since Thomas (1) postulated that both enzyme systems are important in insulin degradation, the transhydrogenase at high insulin concentrations and the protease as "a fine controlling mechanism" when insulin concentrations are low. As shown here, both activities seem to reside in the same particles which quantitatively have the potential to degrade all the insulin made by the animal.

Until the findings reported here it was difficult to explain the presence of the reductase and proteases largely in different cell fractions. Moreover, since both enzyme systems are largely intracellularly located, this raised serious questions regarding the significance of binding of insulin to plasma membranes. The findings presented here may go a long way in solving this puzzle also, for they provide a basis for a possible mechanism wherein by pinocytosis the lysosome will engulf insulin bound to plasma membranes. Whether the lysosomal system herein described may be the system responsible for degradation of insulin under physiological conditions remains to be investigated. Possibly the glutathione-insulin transhydrogenase and protease found in microsomes and cytosol respectively can provide a safeguard against massive doses of insulin.

It is presently believed that most if not all hormones bind to specific hormone receptors; since many bind to cell surface receptors, it seems possible that a large part of hormonal degradation may be carried out by lysosomes,

as suggested by the experiments presented here.

Finally, it is appropriate to note that lysosomes were discovered as a result of the interest of deDuve (10) in insulin.

ACKNOWLEDGEMENTS

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