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EVIDENCE FOR LYSOSOMAL REDUCTION OF CYSTINE RESIDUES

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SUMMARY

Previously reported evidence for the existence of a thiol: protein disulphide oxidoreductase in rat liver lysosomes has been re-examined and ambiguous results obtained. However, incubation of purified rat liver lysosomes with ^{125}I-labelled insulin at pH 5.5 shows that cathepsin D and a thiol-dependent enzyme other than cathepsin B or L are important in its digestion. The latter enzyme is most probably a thiol: protein disulphide oxidoreductase.

INTRODUCTION

Lysosomes are the major site of intracellular protein degradation. Exogenous proteins reach the lysosomes after pinocytosis or phagocytosis and are there digested to the level of free amino acids and dipeptides, which are small enough molecules to escape into the cytosol through the lysosome membrane (1). Endogenous proteins can enter lysosomes by autophagy or crinophagy, and degradation of cytoplasmic proteins may be achieved by their adsorption to the cytoplasmic face of lysosomes and subsequent transfer to the lysosome interior by a process of lysosomal involution (2).

Many proteins contain cystine residues, but little attention has been paid to the fate of these residues within lysosomes. If lysosomes have no mechanism for cleaving the disulphide bond, cystine residues in proteins must always be reduced prior to entry into lysosomes, or intralysosomal proteolysis must take place with cystine residues remaining intact throughout. If the latter, cystine must be able to penetrate across the lysosome membrane. This may be so, although the molecular weight ceiling for permeability of amino acids is around 220 (3) and cystine's molecular weight is 240. Also, evidence from studies on the human inborn error of metabolism cystinosis (4)

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does not support a hypothesis that intact cystine can cross the lysosomal membrane.

If cystine residues can be cleaved within lysosomes, this may occur before, during or after proteolysis. A hydrolytic cleavage to cysteine and cysteine sulphenic acid would parallel the action of other lysosomal enzymes but would be unprecented metabolically. Reduction to cysteine is more probable, but carries the requirement of a reducing agent either generated within or able to penetrate into the lysosomes, and also able to penetrate out in oxidized form.

No glutathione-cystine transhydrogenase could be demonstrated in the lysosomes of human leucocytes (5), but in 1976 the presence of a glutathione-protein transhydrogenase in rat liver lysosomes was reported (6). These results suggest that lysosomes, although unable to reduce free cystine, may be able to reduce cystine residues in proteins. Also that cystinosis may result from the congenital lack of the lysosomal thiol : protein disulphide oxidoreductase (7). We have therefore sought further evidence for the presence of this enzyme activity in rat liver 'Tritosomes' - lysosomes freed from contaminating organelles by manipulating their buoyant density.

MATERIALS AND METHODS

Chemicals. Sodium [^{125}I]iodide (IMS 30, 100 mCi/ml) was from the Radiochemical Centre, Amersham. Pepstatin and leupeptin were from Protein Research Foundation, Osaka. All other chemicals were from Sigma. Bovine insulin was ^{125}I-labelled by the chloramine-T method (8) and excess [^{125}I] iodide was removed by dialysis against 1% NaCl. The labelled insulin was stored at -20°C, under which conditions the acid-soluble radioactivity remained constant at approx. 2% for at least one year. Sulphatolysis (9) of the labelled insulin into A and B chains showed that 88% of the ^{125}I was attached to the A-chain. Formaldehyde-denatured ^{125}I-labelled bovine serum albumin was prepared by the method of Moore et al. (8).

Polypeptide digestion by lysosomal enzymes. Rat liver Tritosomes in sucrose solution (1 mg protein per ml) were prepared by the method of Trouet (10). Polypeptide degradation was measured by monitoring the conversion of ^{125}I-labelled insulin or albumin into fragments soluble in 5% trichloroacetic acid. The reaction mixture consisted of tracer amounts of ^{125}I-labelled insulin (plus 100 µg of unlabelled insulin) or of formaldehyde-denatured ^{125}I-labelled albumin, bovine serum albumin (4 mg), and Tritosomes (100 µl), all in a total volume of 0.9 ml of 0.2M citrate-phosphate buffer containing 1mM reduced glutathione and 0.2% Triton X-100. Incubation was at 37°C and

reactions were stopped by the addition of 20% trichloroacetic acid (0.3 ml). The precipitate formed was collected by centrifugation at 1500 g for 20 min and, after the precipitate had been washed with 5% trichloroacetic acid (1 ml), the combined supernatant and the pellet (dissolved in 2 ml 20% KOH) were separately counted in a Packard Selektronic gamma spectrometer. Insulin digestion products were analysed by Sephadex G-75 chromatography (11).

RESULTS

Grisolia and Wallace (6) report in bare outline only the method used and the results obtained to detect glutathione insulin transhydrogenase in lysosomes. They omit to state the pH of the assay mixture. Using their method we could not detect any transhydrogenase in Tritosomes when incubations were performed at pH 7, and we found the method impossible to use at pH 5, owing to the insufficient solubility of insulin and the rapid oxidation of NADPH even in control tubes. In our opinion the method is inherently unreliable, as the parameter measured, NADPH oxidation, is not necessarily coupled only to insulin reduction. We therefore turned to experiments in which ^{125}I-labelled insulin was incubated with Tritosomes at pH values between 3 and 7.

Fig. 1 shows the pH profile for the release of acid-soluble radioactivity from ^{125}I-labelled insulin incubated at 37°C with Tritosomes. Subsequent experiments were performed at either pH 4, the pH optimum, or pH 5.5, a value closer to the probable intralysosomal pH in vivo (3).

Fig. 2 shows the time-course of the production of acid-soluble radioactivity from ^{125}I-labelled insulin incubated with Tritosomes at pH 4.0 or 5.5. The effects of omitting reduced glutathione and of adding pepstatin, a specific inhibitor for cathepsin D (12), or leupeptin, an inhibitor of cathepsins B and L (12), are also shown. No digestion was seen in incubations without Tritosomes. Sephadex G-75 chromatography of incubation mixtures at 4 h and 12 h indicated a progressive decrease in radioactivity associated with intact insulin and the appearance of a broad unresolved peak corresponding to [^{125}I] iodotyrosine and small labelled peptides. No [^{125}I] iodide was generated during the incubations. The presence or absence

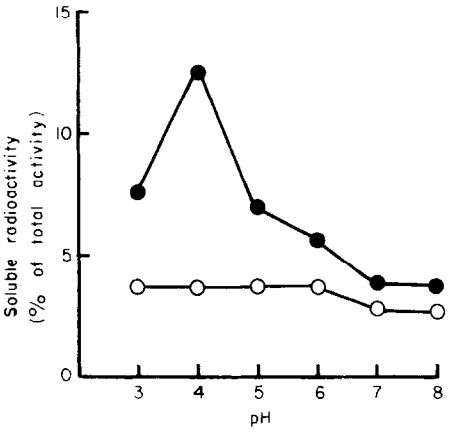

Fig. 1. Production of acid-soluble radioactivity from ^{125}I-labelled bovine insulin incubated for 15 min with rat liver Tritosomes in the presence (●—●) and absence (○—○) of reduced glutathione (1mM). Each value shown has been corrected by subtracting the acid-soluble radioactivity found in an equivalent incubation containing all components of the assay except Tritosomes, and is the average of duplicate measurements.

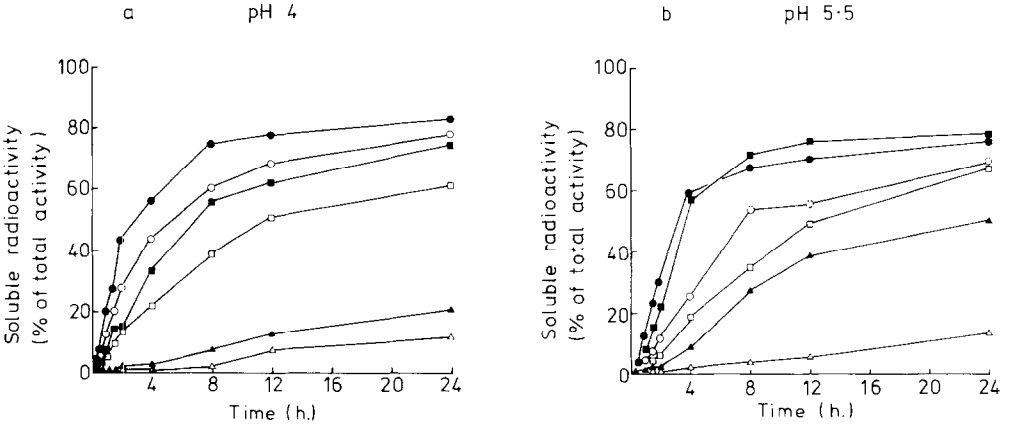

Fig. 2. The effect of proteinase inhibitors on the time-course of production of acid-soluble radioactivity from ^{125}I-labelled bovine insulin incubated with rat liver Tritosomes at pH 4.0 (Fig. 2a) and pH 5.5 (Fig. 2b). The effects of pepstatin (10 µg/ml), with (▲—▲) or without (△—△) reduced glutathione, leupeptin (20 µg/ml), with (■—■) or without (□—□) reduced glutathione, are compared with results obtained without inhibitor, in the presence (●—●) or absence (○—○) of reduced glutathione. Each value shown has been corrected by subtracting the acid-soluble radioactivity found in an equivalent incubation containing all components of the assay except Tritosomes, and is the average of duplicate measurements.

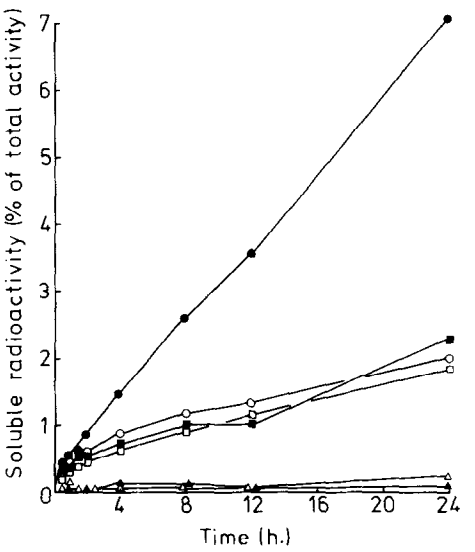

Fig. 3. The effect of proteinase inhibitors on the time-course of production of acid-soluble radioactivity from formaldehyde-denatured ^{125}I-labelled bovine serum albumin incubated with rat liver Tritosomes at pH 5.5. The effects of pepstatin (10 μg/ml), with (▲—▲) or without (△—△) reduced glutathione; leupeptin (20 μg/ml), with (■—■) or without (□—□) reduced glutathione, are compared with results obtained without inhibitor, in the presence (●—●) or absence (○—○) of reduced glutathione. Each value shown has been corrected by subtracting the acid-soluble radioactivity found in an equivalent incubation containing all components of the assay except Tritosomes, and is the average of duplicate measurements.

of glutathione or inhibitors caused quantitative but no major qualitative differences in elution patterns on Sephadex G-75 chromatography.

Since pepstatin inhibited insulin degradation strongly at pH 4.0, but only partially at pH 5.5, and since pepstatin is reported to be less effective at pH values above 5 (13), further experiments were conducted with the same concentration of pepstatin but formaldehyde-denatured ^{125}I-labelled bovine serum albumin as substrate. Fig. 3 shows that production of acid-soluble radioactivity was completely halted by pepstatin and that partial inhibition could be achieved either by leupeptin or the omission of reduced glutathione. Since omitting reduced glutathione did not cause further inhibition in an incubation containing leupeptin, it appears that the requirement for reduced glutathione in the digestion of ^{125}I-labelled albumin relates solely to leupeptin-sensitive proteinases, chiefly cathepsins B and L.

DISCUSSION

Release of acid-soluble radioactivity from ^{125}I-labelled insulin by Tritosomes at pH 4 was strongly inhibited by pepstatin, indicating an important role for cathepsin D. Some inhibition was also observed when reduced glutathione was omitted or when leupeptin was present.

At pH 5.5 strong inhibition was only seen when the presence of pepstatin was combined with omission of reduced glutathione. Pepstatin alone was much less inhibitory, although at the same concentration and pH it completely inhibited digestion of ^{125}I-labelled albumin. Absence of reduced glutathione was in itself only mildly inhibitory. This pattern suggests that release of small fragments from ^{125}I-labelled insulin requires both cathepsin D and a thiol-dependent enzyme. The latter cannot be cathepsin B or L, since leupeptin fails to enhance inhibition by pepstatin in incubations containing reduced glutathione (results not shown), and is therefore most probably a disulphide reducing enzyme.

We showed that 88% of the insulin radiolabel was associated with the A-chain and, since up to 80% of the label was released in acid-soluble form in our experiments, most of this must derive from labelled tyrosine residues (number 14 and/or 19) on the A-chain. Tyrosine-19 is the preferentially labelled residue (14). Release of this residue, or of tyrosine-14, as part of a fragment of lower molecular weight than insulin itself, requires the cleavage of at least two bonds, which could either both be peptide linkages or be one peptide and one disulphide. Our results point clearly to the importance of cathepsin D in the release of acid-soluble radiolabelled fragments from ^{125}I-labelled insulin, and this enzyme does not cleave any bonds in insulin A-chain distal to asparagine-18 (15). Presumably the second cleavage is reduction of the cystine at A-chain residue 20. Possible but less likely is breakage of a peptide bond by some thiol-dependent lysosomal peptidase other than cathepsins B and L.

Our results, both with labelled insulin and labelled albumin, do not contradict the claim (16, 17) that cathepsin B is more important than cathepsin D in the digestion of proteins by lysosomes, since we have been measuring the release of a specific residue in insulin, and not the general release of acid-soluble or ninhydrin-positive material. It may well be that cathepsin B can effectively attack regions of the insulin molecule other than that around tyrosine-19.

ACKNOWLEDGEMENT

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