Role of thiols in degradation of proteins by cathepsins

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The effects of thiols on the breakdown of ^{125}I -labelled insulin, albumin and formaldehyde-treated albumin by highly purified rat liver cathepsins B, D, H and L at pH 4.0 and 5.5 were studied. At both pH values degradation was strongly activated by the thiols cysteamine, cysteine, dithiothreitol, glutathione and 2-mercaptoethanol, and its rate increased with increasing thiol concentration. Preincubation of the protein substrates with 5 mM-glutathione did not affect the rate of degradation by cathepsin D or L, and determination of free thiol groups after incubation of the proteins in the presence of glutathione but without cathepsin showed that their disulphide bonds were stable under the incubation conditions. Sephadex G-75 chromatography of the acid-soluble products of insulin digestion by cathepsin D or L suggested that thiols can reduce disulphide bonds in proteins after limited proteolysis. The resultant opening-up of the protein structure would lead to further proteolysis, so that the two processes (proteolysis and reduction) may act synergistically. By using the osmotic-protection method it was shown that, at a physiological pH, cysteamine, and its oxidized form cystamine, can cross the lysosome membrane and thus may well be the physiological hydrogen donor for the reduction of disulphides in lysosomes. The results are discussed in relation to the lysosomal storage disease cystinosis.

An unsolved problem in the catabolism of proteins within lysosomes concerns cystine residues. It is unlikely that all proteins entering lysosomes have their disulphide linkages reduced before entry, and indeed it has been shown that the intralysosomal breakdown of endogenous and exogenous proteins can contribute to the lysosomal cystine pool in the human inborn error of metabolism cystinosis (Thoene *et al.*, 1977; Thoene & Lemons, 1980). It is also unlikely that cystine (mol.wt. 240) can traverse the lysosome membrane, as the molecular-weight ceiling for permeability of amino acids is generally around 220 (Schulman & Bradley, 1970; Lloyd, 1971, 1973; Reijngoud & Tager, 1977). This conclusion also receives support from the intralysosomal accumulation of cystine in cystinosis (Oshima *et al.*, 1976), although it could be that the defect in that disease alters the permeability of the lysosome membrane. If cystine cannot escape from lysosomes, it is necessary to postulate the existence of some system or systems capable of cleaving the disulphide bond within the lysosome. Reduction of cystine residues may occur before or during proteolysis or at the level of free cystine. It has been suggested that, by analogy with the cytosol, lysosomes may contain enzymes that catalyse

thiol:disulphide interchanges and that cystinosis may result from a defective intralysosomal reductase (Lloyd & Williams, 1978). Tietze *et al.* (1972) failed to find a lysosomal cystine:glutathione transhydrogenase in human leucocytes, but evidence for the existence of a glutathione:protein disulphide transhydrogenase in rat liver lysosomes was presented by Grisolia & Wallace (1976). Griffiths & Lloyd (1979) re-examined this evidence and obtained ambiguous results. They found, however, that the release of trichloroacetic acid-soluble radioactivity from ^{125}I -labelled bovine insulin, when incubated with broken rat liver tritosomes, was accelerated by glutathione, halted by pepstatin, an inhibitor of cathepsin D, but unaffected by leupeptin, an inhibitor of the thiol-dependent cathepsins. Since the activity of cathepsin D is unaffected by thiol reagents, it was concluded that both cathepsin D and a leupeptin-insensitive but thiol-dependent enzyme, most probably a disulphide-reducing enzyme, are needed for the rapid release of acid-soluble radioactivity from ^{125}I -labelled insulin. In the present paper we have sought to test this hypothesis by determining whether thiols stimulate the degradation of cystine-containing polypeptides by highly purified cathepsins B, D, H and L and whether reduction of

disulphide bonds occurs before or during proteolysis.

Any reduction of disulphide within lysosomes, even if non-enzymic, poses problems relating to the permeability of the lysosomal membrane, for it requires that the hydrogen donor must be able in its reduced form to penetrate from the cytosol into the lysosomes and in its oxidized form to pass out of the lysosomes into the cytosol. Thoene *et al.* (1976) demonstrated that the abnormal high free (non-protein) cystine pool of cultured cystinotic skin fibroblasts is decreased by over 90% in 1 h by 1 mM-cysteamine (or cystamine), and they proposed a model that accounts for the observed results. In this model, cysteamine enters the lysosome and reacts with free cystine, producing one molecule of mixed disulphide and one molecule of cysteine. Reaction of the mixed disulphide with cysteamine produces another molecule of cysteine and one molecule of cystamine. Cystamine leaves the lysosome and is then reduced to cysteamine in the cytoplasm by reaction with glutathione. Since cysteamine is a normal intracellular metabolite (Poulsen & Ziegler, 1977), this thiol could also be the physiological hydrogen donor required for the intralysosomal reduction of cystine residues in normal cells.

This proposed scheme requires that cysteine, cysteamine and cystamine should be able to diffuse freely in and out of the lysosome. It is likely that they can, since their molecular weights are all well below 200, but it is important to obtain direct information. We have therefore used the osmotic-protection method (Lloyd, 1969) to investigate the ability of these substances to cross the lysosome membrane.

A preliminary report of some of this work has been published (Kooistra *et al.*, 1981).

Materials and methods

Biochemical reagents

Na¹²⁵I (IMS.30) was from The Radiochemical Centre, Amersham, Bucks., U.K. Pepstatin and leupeptin were from the Protein Research Foundation, 476, Ina, Minoh-shi, Osaka 562, Japan. Bovine pancreas insulin (I 5500), bovine serum albumin (A 7638), lactoperoxidase (L 7129) and Sephadex G-75-40 were from Sigma Chemical Co., Poole, Dorset, U.K. *p*-Nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside were from Koch-Light, Colnbrook, Bucks., U.K. Purified cathepsins B (EC 3.4.22.1), D (EC 3.4.23.5), H (EC 3.4.22.16) and L (EC 3.4.22.15) from rat liver were prepared and supplied by Dr. Heidrun Kirschke [Physiologisch-Chemisches

Institut der Martin-Luther Universität Halle-Wittenberg, 402 Halle (Saale), German Democratic Republic]. All other reagents used were analytical grade.

Insulin was iodinated by the lactoperoxidase method, as described in detail by Hamlin & Arquilla (1974), and excess [¹²⁵I]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% of total radioactivity for at least 6 months. Sulphitolysis (Varandani, 1966) of the labelled insulin into A and B chains showed that 95% of the iodine was attached to the A chain. Hamlin & Arquilla (1974) determined the iodine distribution in the A chain as 80% in tyrosine-A14 and 20% in tyrosine-A19. [¹²⁵I]-labelled albumin was prepared by the chloramine-T procedure, as described for ribonuclease (Kooistra *et al.*, 1979). Formaldehyde denaturation of [¹²⁵I]-labelled albumin was by the method of Moore *et al.* (1977).

Preparation of a lysosome-enriched suspension

Lysosome-enriched fractions of rat liver were prepared by the method of Lloyd (1969), with 0.25 M-mannitol replacing sucrose as the homogenizing medium. The lysosome fraction so prepared is contaminated with other cell particles (particularly mitochondria and peroxisomes), but the preparation time (approx. 1 h) is shorter than that of a more rigorous purification and the lysosomes are thus more likely to retain their functional integrity. The lysosome-enriched pellet was gently re-suspended in a volume of 0.25 M test-compound solution equal to five times the original liver weight. Up to this point all operations were carried out at 4°C. The lysosome fraction was always used immediately after preparation.

Measurement of the permeability of rat liver lysosomes to low-molecular-weight compounds

The lysosomal suspension in 0.25 M test-compound solution was adjusted to pH 5.0, 6.0 or 7.4, with NaOH or HCl. This avoided the use of a buffer solution, which would have increased the osmolarity of the suspension, and thus increased the osmotic protection given to the lysosomes. These suspensions were then incubated in a water bath at 25°C for 2 h. At various time intervals the activity of *N*-acetyl- β -D-glucosaminidase was determined in the absence (free activity) and presence (total activity) of 0.1% (v/v) Triton X-100, and the results are expressed in terms of free activity as a percentage of the total. Both the free and total activity were determined at each time point. All assays contained 0.20 M-mannitol plus 0.05 M of the compound in which the granules had been suspended. The 0.25 M solutions of cysteamine were kept under nitrogen to minimize

the possible oxidation to cystamine. No significant oxidation of any of the other substances was envisaged.

The theory behind the osmotic-protection method (see Lloyd, 1969) is that lysosomes remain osmotically stable when suspended in an iso-osmotic solution of an impermeant solute, thus maintaining the latency of lysosomal enzymes. In similar solutions of permeant compounds, however, osmotic balance is lost as solute penetrates the membrane and, as a result, water is drawn into the lysosomes. The resultant hydrostatic pressure disrupts the lysosome membrane. By measuring the rise in 'free' activity of lysosomal enzymes (an indication of the extent of lysosomal breakage), an indirect measure of the rate and extent of test-compound uptake is obtained.

Assays

The standard incubation mixture for measurement of the degradation of ^{125}I -labelled protein contained: 0.2 M-sodium formate (pH 4) or 0.2 M-sodium acetate (pH 5.5), 5 mM-EDTA, 0, 1 or 5 mM-glutathione (or other thiol), 100 μg of ^{125}I -labelled protein/ml and 10 μg of cathepsin B/ml, 3.3 μg of cathepsin D/ml, 10 μg of cathepsin H/ml or 1.3 μg of cathepsin L/ml in a total volume of 0.5 ml. Incubations were for 5 h at 37°C and were terminated by the addition of 0.1 ml of 5% (w/v) bovine serum albumin and 0.2 ml of 20% (w/v) trichloroacetic acid. Acid-soluble radioactivity, used as a measure of protein hydrolysis, was corrected by subtracting the acid-soluble radioactivity found in an equivalent incubation containing all compounds of the assay except enzyme. The non-enzymic release of acid-soluble radioactivity from radiolabelled albumin was negligible, and that from radiolabelled insulin was about 0.1% at pH 4 and 1.0–1.7% at pH 5.5, with 5 mM-glutathione, and 0–0.1% at pH 4 and 5.5 without glutathione. When required, pepstatin (10 μg /ml) or leupeptin (20 μg /ml) was included in the incubation mixture. All assays were performed in duplicate. Insulin digestion products were analysed by Sephadex G-75 chromatography (Varadani & Shroyer, 1973).

The assay method for *N*-acetyl- β -D-glucosaminidase was essentially that of Barrett & Heath (1977), with *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside or 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside as substrate. The assay was done at 25°C, necessitating the use of an incubation time of 15 min.

Free thiol groups were determined spectrophotometrically after reaction with 5,5'-dithiobis-(2-nitrobenzoic acid), essentially by the method of Ellman (1959). For calculation of the thiol-group content, a molar absorption coefficient of 13 600 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 412 nm was used.

Results

Cathepsins B, D, H and L could each release acid-soluble radioactivity from ^{125}I -labelled insulin, albumin and formaldehyde-denatured albumin. In agreement with earlier studies (Ansorge *et al.*, 1977; Kirschke *et al.*, 1977), cathepsin L was by far the most active thiol-dependent proteinase, and also showed a greater specific activity towards the protein substrates than did cathepsin D. Pepstatin, a specific inhibitor of cathepsin D (Barrett, 1980), completely halted degradation of ^{125}I -labelled insulin by cathepsin D, but had no effect on that by cathepsins B, H or L. Leupeptin, an inhibitor of cysteine proteinases (Barrett, 1980), did not affect digestion of labelled insulin by cathepsin D, but strongly inhibited that by cathepsin B, H and L. These inhibitor studies demonstrate that the cathepsin D preparation is not contaminated with the thiol-dependent cathepsins, and vice versa.

Table 1 shows the effects of glutathione concentration and pH on the degradation of the three labelled proteins by cathepsins B, D, H and L. Digestion increased with increasing thiol concentration at both pH 4.0 and 5.5. The optimum pH was related to both enzyme and substrate. In general, cathepsins B and D were most active at pH 4.0, and cathepsin H at pH 5.5, whereas the activity of cathepsin L was about the same at both pH values. The conformational instability of the albumin molecule at pH 4.0 compared with pH 5.5 (see Huisman *et al.*, 1973) might explain why this protein is much more susceptible to degradation at the lower pH.

Studies in which 5 mM-cysteamine, -cysteine, -dithiothreitol or -2-mercaptoethanol replaced 5 mM-glutathione in the incubation mixture showed that all five thiols stimulated protein breakdown to about the same extent.

Fig. 1 shows the profiles of radioactivity on a Sephadex G-75 column of the acid-soluble digestion products of ^{125}I -labelled insulin on incubation with cathepsin D or L. Incubation of radiolabelled insulin with cathepsin D at pH 4 without glutathione led to two peaks, one apparently smaller and one apparently larger than insulin A-chain. The presence of 5 mM-glutathione in the reaction mixture resulted in an elution pattern indicative of several degradation products. Qualitatively similar elution profiles were observed when the incubations were at pH 5.5. Chromatography of the acid-soluble products of radiolabelled insulin incubated with cathepsin L in the presence of 5 mM-glutathione at pH 5.5 (or 4.0) showed only one main peak, apparently a fragment much smaller than A-chain. The fragment is not [^{125}I]iodotyrosine, which in this system is eluted even after potassium bichromate.

Preincubation of insulin, albumin or denatured

Table 1. *Effect of thiol and pH on protein degradation by cathepsins B, D, H and L*

Degradation of ^{125}I -labelled bovine insulin, bovine serum albumin and formaldehyde-denatured bovine serum albumin by cathepsins B, D, H and L was determined as described in the Materials and methods section. The incubation mixture contained 5 mM-EDTA, 100 μg of ^{125}I -labelled test protein/ml and 10 μg of cathepsin B/ml, 3.3 μg of cathepsin D/ml, 10 μg of cathepsin H/ml or 1.3 μg of cathepsin L/ml in a total volume of 0.5 ml, with various concentrations of glutathione. Incubations were for 5 h at 37°C and degradation was measured as radioactivity soluble in 5% trichloroacetic acid. Each value shown has been corrected by subtracting the acid-soluble radioactivity found in an equivalent incubation containing all components of the assay except cathepsin, and is the average of duplicate measurements.

Incubation conditions	Degradation (% of total radioactivity)					
	Insulin		Albumin		Denatured albumin	
	pH 4.0	pH 5.5	pH 4.0	pH 5.5	pH 4.0	pH 5.5
Cathepsin B	4.3	2.4	1.3	0.1	1.6	0.0
Cathepsin B + 1 mM-glutathione	18.6	4.2	4.0	0.3	4.1	0.4
Cathepsin B + 5 mM-glutathione	24.9	9.8	7.8	0.6	8.0	0.9
Cathepsin D	19.0	1.6	8.7	0.2	2.5	0.0
Cathepsin D + 1 mM-glutathione	31.3	11.8	12.7	1.0	3.4	0.6
Cathepsin D + 5 mM-glutathione	40.9	27.3	17.6	1.4	5.7	2.1
Cathepsin H	1.4	8.2	0.3	0.1	0.1	0.0
Cathepsin H + 1 mM-glutathione	2.4	39.1	0.6	0.2	0.5	0.4
Cathepsin H + 5 mM-glutathione	3.7	41.6	0.9	0.3	0.9	0.6
Cathepsin L	7.8	7.4	19.3	2.0	5.8	2.0
Cathepsin L + 1 mM-glutathione	46.3	60.2	63.9	5.0	52.4	22.0
Cathepsin L + 5 mM-glutathione	60.4	63.2	66.4	6.4	55.9	37.2

albumin with 5 mM-glutathione for 5 h before addition of cathepsin B, D, H or L did not increase the rate of degradation above that seen without preincubation of protein substrate with thiol (results not shown). Table 2 shows that under incubation conditions, in the absence of cathepsin, the disulphide bonds of the three proteins are not reduced by glutathione, but that in the presence of 8 M-urea some of the disulphide bonds in insulin and albumin are cleaved.

Fig. 1. *Sephadex G-75 chromatography of trichloroacetic acid-soluble digestion products from the action of cathepsin D or L on ^{125}I -labelled insulin* ^{125}I -labelled insulin was incubated for 5 h with cathepsin D at pH 4.0 in the absence (a) or presence (b) of 5 mM-glutathione, or with cathepsin L at pH 5.5 with 5 mM-glutathione (c), as described in the Materials and methods section. The resulting acid-soluble digestion products were analysed by chromatography on a Sephadex G-75 (superfine grade) column (3.2 cm $\times$ 88.3 cm) as described by Varadani & Shroyer (1973). The column was eluted with 50% (v/v) acetic acid at a rate of 12 ml/h, the fraction size being 3.9 ml. Relative concentrations of radioactivity are expressed as percentages of recovered radioactivity. Recoveries were at least 90%. The vertical marks show the positions of reference compounds: I, insulin; II, insulin B chain; III, insulin A chain; IV, V_t (total volume of the packed bed, as determined by the elution of potassium bichromate).

Table 2. *Determination of free thiol groups in insulin, albumin and denatured albumin after incubation under assay conditions in the absence or presence of 8 M-urea*

Free thiol groups were determined by the spectrophotometric method of Ellman (1959). Insulin (2.5 mg) or albumin (5.0 mg) were dissolved in a 3.1 ml incubation mixture without cathepsin (see the Materials and methods section), in the absence or presence of 8 M-urea. Samples (1.0 ml) were added to 1.0 ml of deaerated 20% trichloroacetic acid, containing 4 M-NaCl to facilitate precipitation. The precipitate was washed three times with deaerated 10% trichloroacetic acid, containing 2 M-NaCl, and dissolved in 3.0 ml of a deaerated solution containing 0.5 M-Tris/acetate buffer, pH 8.0, 8 M-urea and 0.165 mM-5,5'-dithiobis-(2-nitrobenzoic acid). After 60 min at room temperature the A_{412} was determined. Each value is the mean of three experiments, with a maximal deviation from the mean of 0.2.

Incubation conditions	No. of free thiol groups per protein molecule					
	Insulin		Albumin		Denatured albumin	
	pH 4.0	pH 5.5	pH 4.0	pH 5.5	pH 4.0	pH 5.5
No glutathione	0.0	0.0	0.3	0.3	0.4	0.1
1 mM-glutathione	0.0	0.0	0.3	0.3	0.2	0.3
5 mM-glutathione	0.0	0.0	0.5	0.3	0.2	0.1
No glutathione + 8 M-urea	0.0	0.0	0.4	0.4	0.3	0.2
1 mM-glutathione + 8 M-urea	0.0	0.2	0.9	1.8	0.2	0.1
5 mM-glutathione + 8 M-urea	0.2	1.4	2.6	2.8	0.1	0.1

Table 3. *Change in free N-acetyl- β -D-glucosaminidase activity of rat liver lysosomes incubated in various compounds*
A lysosome-enriched pellet was resuspended in a 0.25 M solution of the test compound. The suspension was adjusted to the pH shown, and then incubated at 25°C. At the time shown, the free and total N-acetyl- β -D-glucosaminidase activity was measured, as described in the Materials and methods section. All values of the free activity (expressed as % of the total activity) are means $\pm$ S.E.M. of the stated numbers of experiments.

Compound (mol.wt.)	pH	No. of expts.	Incubation time at 25°C (min)				
			0	30	60	90	120
Mannitol (182)	5.0	3	9.4 $\pm$ 0.8	13.1 $\pm$ 1.1	17.4 $\pm$ 0.5	25.0 $\pm$ 3.8	35.2 $\pm$ 4.9
	6.0	4	9.3 $\pm$ 1.0	11.5 $\pm$ 1.3	14.1 $\pm$ 1.4	16.8 $\pm$ 1.0	21.1 $\pm$ 1.6
	7.4	4	8.4 $\pm$ 0.5	11.2 $\pm$ 0.9	18.2 $\pm$ 2.4	22.1 $\pm$ 1.3	28.9 $\pm$ 2.3
L-Serine (105)	5.0	3	10.5 $\pm$ 1.3	12.3 $\pm$ 2.4	24.1 $\pm$ 9.4	34.6 $\pm$ 10.0	58.5 $\pm$ 16.2
	6.0	3	11.1 $\pm$ 2.2	13.1 $\pm$ 2.4	20.2 $\pm$ 5.4	28.0 $\pm$ 6.4	43.2 $\pm$ 10.2
	7.4	3	9.4 $\pm$ 0.6	13.0 $\pm$ 1.5	28.8 $\pm$ 2.1	40.0 $\pm$ 2.1	54.7 $\pm$ 3.7
L-Cysteine (121)	5.0	6	11.9 $\pm$ 1.2	17.0 $\pm$ 2.0	48.1 $\pm$ 7.6	79.9 $\pm$ 7.9	93.6 $\pm$ 4.5
	6.0	5	11.1 $\pm$ 1.5	15.7 $\pm$ 1.4	37.4 $\pm$ 8.5	54.7 $\pm$ 12.2	75.1 $\pm$ 11.3
	7.4	7	11.1 $\pm$ 1.3	16.0 $\pm$ 2.4	29.5 $\pm$ 6.6	53.3 $\pm$ 10.8	84.1 $\pm$ 8.3
Cysteamine (77)	5.0	3	15.4 $\pm$ 4.2	48.8 $\pm$ 7.9	66.4 $\pm$ 2.3	76.1 $\pm$ 3.8	77.8 $\pm$ 0.6
	6.0	3	15.5 $\pm$ 1.7	22.4 $\pm$ 0.6	43.4 $\pm$ 3.4	52.9 $\pm$ 3.3	48.4 $\pm$ 4.2
	7.4	3	20.9 $\pm$ 4.5	54.0 $\pm$ 7.6	51.2 $\pm$ 1.4	60.9 $\pm$ 5.0	86.7 $\pm$ 10.7
Cystamine (152)	5.0	3	12.1 $\pm$ 1.2	15.6 $\pm$ 1.4	17.0 $\pm$ 2.4	19.4 $\pm$ 1.5	20.9 $\pm$ 1.8
	6.0	3	11.2 $\pm$ 1.4	14.3 $\pm$ 0.9	15.7 $\pm$ 1.3	18.8 $\pm$ 0.4	21.0 $\pm$ 2.5
	7.4	3	11.6 $\pm$ 1.0	26.2 $\pm$ 4.4	47.8 $\pm$ 5.8	64.1 $\pm$ 3.3	74.9 $\pm$ 1.1

Apparently, the disulphide bonds of insulin and albumin are resistant to reduction, unless the protein structure is first opened up by, for example, a denaturing agent such as urea. In the absence of glutathione, albumin was shown to contain 0.3–0.5 free thiol group per molecule. This is in agreement with data of Hird (1962).

Table 3 shows the time-course of osmotic lysis of lysosomes suspended in 0.25 M solutions of various

compounds, measured by the increase in the free activity of N-acetyl- β -D-glucosaminidase. Suspension in mannitol led to relatively little increase in the percentage free enzyme activity during the course of the incubations, confirming the observations of Lloyd (1969) that it is unable to penetrate the lysosome membrane, despite its low molecular weight, 182. Incubation in 0.25 M-serine (the O analogue of cysteine) caused the free activity to

increase to 40–60% in 2 h, data consistent with those for glycine, alanine and valine (Lloyd, 1971). At all three pH values, cysteine (mol.wt. 121) penetrated the lysosomal membrane more rapidly than did serine (mol.wt. 106), the most rapid increase in free activity occurring at pH 5.0. These results are consistent with the conclusion of Lloyd (1971) that conformation and/or charge are as important as molecular weight in determining the ability of a compound to cross the lysosome membrane. Incubation of lysosomes in cysteamine caused an increase in the free enzyme activity at all three pH values, although at pH 6.0 the increase reached a plateau for no obvious reason after 60–90 min of incubation. Cystamine exhibited strongly pH-dependent behaviour in its ability to preserve lysosomal integrity. At pH 5.0 and 6.0 cystamine did not permeate the membrane of the lysosomes, and was in fact better than mannitol for preserving lysosomal integrity. At pH 7.4, however, the percentage of free glucosaminidase activity rose quickly.

Discussion

We have shown that the degradation of ^{125}I -labelled insulin, albumin and formaldehyde-denatured albumin by cathepsins B, D, H and L is strongly thiol-dependent at both pH 4.0 and 5.5. This result is not unexpected for those cathepsins (B, H and L) whose catalytic activity is thiol-dependent, but in the case of cathepsin D it cannot be explained by enzyme activation. Griffiths & Lloyd (1979), finding thiol-stimulation of insulin catabolism by the cathepsin D activity of tritosomes, postulated the presence in lysosomes of a thiol:protein disulphide oxidoreductase. That explanation is not available in the present experiments, since the enzyme used was a highly purified cathepsin D.

The stimulant action of thiols was not an effect on the intact protein molecules: preincubation of the proteins with 5 mM-glutathione did not affect the rate of degradation by the proteinases, and determination of free thiol groups in the proteins after incubation under assay conditions in the absence of cathepsin showed that the disulphide bonds of the protein molecules were stable under such conditions. Similar results were obtained by Davidson & Hird (1967), who found that glutathione failed to reduce the disulphide bonds in many native proteins, when incubated under physiological conditions (pH 7.4; 6.25 mM-glutathione).

Table 2 shows, however, that the non-enzymic reduction of disulphide bonds by glutathione is greatly accelerated in the presence of 8 M-urea, particularly at the higher pH value and glutathione concentration. Sephadex G-75 chromatography of the acid-soluble digestion products of ^{125}I -labelled insulin (Fig. 1) suggests that thiols can also reduce

disulphide bonds in proteins after partial hydrolysis. The radiolabel released in acid-soluble form in our experiments must derive almost entirely from labelled tyrosine residues A14 and, to a much lesser extent, A19. Release of tyrosine-A14, as part of an acid-soluble fragment, requires the cleavage of at least two bonds, which could either both be peptide linkages or be one peptide and one disulphide. Cathepsin D can cleave the following bonds in the A-chain: Leu-13–Tyr-14, Tyr-14–Gln-15, Leu-16–Glu-17, Glu-17–Asn-18 (Schwabe & Sweeney, 1972), and thus is perfectly capable of producing small radiolabelled peptides without cleavage of a disulphide bond. However, reduction of the cystine at A-chain residue 7 and/or 20 would lead to many more acid-soluble fragments, as indeed were found (compare Figs. 1a and 1b). Katunuma *et al.* (1981) found that cathepsin L shows preference for the Glu-4–Gln-5, Tyr-14–Gln-15, Gln-15–Leu-16 and Glu-17–Asn-18 bonds of insulin A-chain. Release of fragments much smaller than A-chain (Fig. 1c) is therefore only possible after reduction of the Cys-A7–Cys-B7 disulphide bond, as breakage of the Asn-3–Gln-4 or the Glu-13–Ala-14 peptide bonds of the B chain (Kirschke *et al.*, 1977) would produce larger fragments. These findings are in agreement with studies by Davidson & Hird (1967), who demonstrated that, after hydrolysis of fewer than 10% of the peptide bonds of the proteins investigated, a marked increase in the rate of reduction of the disulphide bonds by glutathione occurred. In similar studies Axelsson & Mannervik (1980) showed that a thioltransferase (thiol:disulphide oxidoreductase) from rat liver cytosol catalysed the reduction by glutathione of disulphide bonds in proteins, provided that their conformation had been disrupted by limited proteolysis or by urea treatment. They further showed that the presence of thioltransferase did not increase the number of disulphide bonds reduced above the number reduced by glutathione alone.

The stimulating effect of thiols on the degradation of the protein substrates by cathepsins might thus be explained as follows: first, thiols activate the thiol-dependent cathepsins B, H and L, and secondly, thiols can reduce cystine residues after partial catheptic proteolysis; the resultant opening-up of the protein structure would lead to further proteolysis, so that the two processes (proteolysis and reduction) act synergistically. It is no longer necessary to postulate a lysosomal thiol:protein-disulphide oxidoreductase to explain the stimulating effect of glutathione on the degradation of insulin by the cathepsin D activity of broken tritosomes (Griffiths & Lloyd, 1979).

As explained in the introduction, the permeability properties of the lysosome membrane probably preclude glutathione or cysteine from acting as the

hydrogen donor for intralysosomal reduction of cystine residues. Furthermore, if cysteamine is the physiological reducing agent, cysteine, cysteamine and cystamine must all be able to traverse the lysosome membrane. The present investigation indicates that, at a physiological pH (7.4), all three compounds cross this membrane rapidly. It may be proposed, as a working hypothesis, that cytoplasmic cysteamine enters the lysosome and participates in protein digestion by reducing cystine residues, either directly or with intralysosomal glutathione as an intermediate. Cystamine re-enters the cytoplasm and is reduced, regenerating cysteamine, by a reaction probably mediated by glutathione (Jocelyn, 1972), either non-enzymically or via glutathione reductase (Tietze, 1972).

The primary defect in cystinosis has not so far been identified, but could plausibly be a metabolic lesion that results in a low cytoplasmic concentration of cysteamine (Schulman, 1979). Exogenous cysteamine depletes accumulated cystine in cystinotic fibroblasts (Thoene *et al.*, 1976), and the present results support the view that cysteamine is the physiological hydrogen donor for intralysosomal reduction of cystine residues. Although there is some indirect evidence against the hypothesis that cystine accumulates in cystinosis because of a decreased availability of cysteamine, direct measurements of cysteamine concentrations in normal and cystinotic cells have not so far proved possible (Schulman, 1979). If the hypothesis is found to be correct, cystinosis would be the only known lysosomal storage disease not involving any deficiency in lysosomal enzymes.

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