Properties of the Membrane Proteins of Rat Liver Lysosomes

THE MAJORITY OF LYSOSOMAL MEMBRANE PROTEINS ARE EXPOSED TO THE CYTOPLASM

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(Received 17 February 1978)

Rat liver lysosomes were lysed and subfractionated by differential centrifugation through 0.2M-NaCl to yield a membranous pellet. This membrane fraction contains less than 20% of the lysosomal protein, adenosine triphosphatase activity of about 1.2 μ mol/min per mg of protein, 120nmol of thiol groups/mg of protein and at least 16 protein and glycoprotein bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The gel patterns of membranes isolated from lysosomes after treatment with (1) [¹²⁵I]iodide-hydrogen peroxide-lactoperoxidase, (2) toluene 2,4-di-isocyanate-activated bovine serum albumin, (3) trypsin and (4) subtilisin indicate that most of the membrane proteins are exposed to the cytoplasm. These exposed proteins are candidates for intracellular receptors which recognize either substances that are to be degraded or vesicles containing those substances.

Since the discovery of lysosomes (de Duve *et al.*, 1955; de Duve & Wattiaux, 1966), their soluble hydrolases have been shown to have an important role in catabolism of extracellular material (Mego *et al.*, 1972; Gregoriadis, 1975) and a probable role in catabolism of intracellular material (Neely & Mortimore, 1974; Segal, 1975; Dean, 1975; Ray *et al.*, 1976). A number of lysosomal storage diseases attest to the necessity of catabolism (O'Brien *et al.*, 1971), and rapid protein catabolism affords metabolic flexibility (Schimke, 1975; Goldberg & St. John, 1976; Conde & Scornik, 1976; Scornik *et al.*, 1978). Yet catabolism must be preceded by entry into lysosomes of the substances that are to be degraded. Very little is known about the entry mechanism (Poole, 1971; Silverstein *et al.*, 1977), especially as pertains to the lysosomal membrane. Previous studies of this membrane have concerned lipid and carbohydrate compositions (Henning *et al.*, 1970, 1973; Henning & Stoffel, 1973; Thinès-Sempoux, 1973; Milsom & Wynn, 1973). However, little attention has been given to the protein composition of the membrane (Wattiaux-de Coninck & Wattiaux, 1969; Kaulen *et al.*, 1970; Schneider, 1977). As part of an effort to understand the molecular basis of entry into the lysosome, we have focused on lysosomal membrane proteins.

Abbreviation used: ATPase, adenosine triphosphatase.

Experimental

Preparation of lysosomes

Male albino rats weighing 150–300g were purchased from Charles River, Wilmington, MA, U.S.A., and were housed in quarters which provided a 12h light–dark cycle daily and food and water *ad libitum*. Rats were treated with Triton WR-1339 (Trouet, 1964) and, after 4 days, liver lysosomes were isolated by flotation through a sucrose gradient (Leighton *et al.*, 1968). Purity was assessed by measuring glucosaminidase and protein as described previously (Schneider, 1977). Lysosomes prepared by flotation gradient centrifugation are intact as evidenced by the latency of lysosomal enzymes, e.g. glucosaminidase was always found to be 80–90% latent.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Polyacrylamide gels (7.5%) were cast, and samples were electrophoresed as described by Weber & Osborn (1969) with the following exception. A two-step denaturation procedure gave more reproducible results and was important in minimizing cathaptic activity: the membrane proteins were thoroughly dispersed in 5% sodium dodecyl sulphate/2mM-N-ethylmaleimide by bath sonication for 5min at room temperature in a Bransonic 12 apparatus and denatured by heating at 100°C for 2min. A second

identical heating in the presence of 5% β -mercaptoethanol afforded complete dissociation. Total protein was detected by staining with Coomassie Blue, and glycoprotein by staining with periodic acid/Schiff reagent (Zacharius *et al.*, 1969). Densitometric records were obtained by using a Gelman Digiscreen Scanner.

Thiol determinations

Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] was used to measure protein thiol groups (Habeeb, 1972). In all cases sodium dodecyl sulphate and EDTA were also present.

[125 I]Iodination

Lactoperoxidase-catalysed iodination was performed by using Na^{125}I and H_2O_2 (Gates *et al.*, 1975). Fresh lysosomes were mixed with 50 mM-Mes (4-morpholine-ethanesulphonic acid), pH 6, 1 mM-EDTA, 0.5 mM-KI, 0.2 mM- H_2O_2 , 1 $\mu\text{mol}/\text{min}$ activity of lactoperoxidase, 14% (w/w) sucrose and Na^{125}I . The mixture was stirred gently at room temperature for 5 min. After iodination, the mixture was diluted with cold 45% (w/w) sucrose/1 mM-tyrosine, and intact lysosomes were reisolated by flotation gradient centrifugation.

Cross-linking with activated bovine serum albumin

Bovine serum albumin was activated by reaction with toluene-2,4-di-isocyanate (Schick & Singer, 1961), and activated albumin was separated from unchanged di-isocyanate by gel filtration through a column (1.5 cm $\times$ 30 cm) of Sephadex G-75, eluted with 50 mM-Tris/HCl (pH 7)/200 mM-NaCl. The cross-linking reaction with lysosomes was carried out essentially as described by Eytan & Schatz (1975), at pH 8, 30°C, in 14% (w/w) sucrose for 1 h with gentle stirring. Intact lysosomes were then reisolated by flotation gradient centrifugation.

Proteolytic digestion

Lysosomes were incubated with 10 μg of trypsin/mg of lysosomal protein in the presence of 1 mM-

CaCl_2 and 14% (w/w) sucrose at 37°C for the indicated times. Proteolysis was arrested by addition of 20 μg of soya-bean trypsin inhibitor/10 μg of trypsin. Then intact lysosomes were reisolated by flotation gradient centrifugation. Subtilisin (proteinase VIII; Sigma, St. Louis, MO, U.S.A.) was mixed with lysosomes in 14% sucrose to give 10 $\mu\text{g}/\text{mg}$ of lysosomal protein. The pH was raised to 10 by addition of Tris to 20 mM. After 5 min at 37°C, the solution was diluted 10-fold with cold 45% (w/w) sucrose and intact lysosomes were reisolated by flotation gradient centrifugation.

ATPase assay

ATPase activity was measured in 20 mM-potassium 4-morpholinopropanesulphonate (pH 7)/50 mM-KCl/0.6 mg of bovine serum albumin/ml/1 mM-MgATP at 37°C for 10 min. The reaction was terminated with 2.7% trichloroacetic acid, and the amount of liberated P_i was determined (Chen *et al.*, 1956).

Preparation of plasma membrane

Plasma membrane enriched in 5'-nucleotidase was isolated from rat liver as described (Ray, 1970). Purification was more than 20-fold on the basis of 5'-nucleotidase activity.

Results

Isolation of lysosomal membranes

In lieu of a marker enzyme activity for the lysosomal membrane we have used, as our prime criterion for a pure membrane fraction, the absence of soluble enzyme activity, i.e. glucosaminidase, in a pellet obtained by centrifugation of disrupted lysosomes at 100000g for 40 min. Table 1 shows that the pellet fraction was free of glucosaminidase when salt was included in the centrifugation medium. Therefore membranes were routinely prepared from lysosomes by dilution of ruptured lysosomes with a buffered salt solution and centrifugation to obtain pelleted membranes which were subsequently washed once with salt solution.

Table 1. Effects of sucrose and salt on preparation of lysosomal membranes

Liver lysosomes, isolated from Triton-treated rats (Leighton *et al.*, 1968), were frozen, thawed and diluted with an equal volume of either 0.25 M-sucrose or 0.2 M-NaCl/50 mM-Tris/ SO_4 (pH 8)/1 mM-EDTA ('salt'). After centrifugation at 35000 rev./min for 40 min in a 50Ti Spinco rotor the supernatants were decanted. The pellets were resuspended in either sucrose or salt solution with a glass/Teflon homogenizer and re-centrifuged. One unit is 1 $\mu\text{mol}/\text{min}$.

Centrifugation	Glucosaminidase (munits)			Protein (mg)		
	Supernatant		Membrane	Supernatant		Membrane
	1	2		1	2	
medium						
Sucrose	3.99	0.22	0.82 (16%)	2.15	0.26	1.60 (40%)
Salt	4.14	0.44	0.06 (1.3%)	2.59	0.45	0.60 (16%)

Table 2. *ATPase activity and thiol content of lysosomal membranes*

Lysosomal membranes and supernatants were separated by using 'salt' as in Table 1. ATPase activity and thiol content were measured as described in the Experimental section.

Subfraction	ATPase activity ($\mu\text{mol}/\text{min}$ per mg of protein)	Thiol content (nmol of SH/mg of protein)
Supernatants	0.11	53
Membrane	1.17	119

Fig. 1. *Analysis of lysosomal membranes and plasma membranes by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis* Lysosomes (L), lysosomal membranes (LM) and plasma membranes (PM) were prepared, denatured and electrophoresed as described in the Experimental section. The amount of sample was 0.025, 0.050 and 0.025 mg of protein respectively. Coomassie Blue-stainable bands number at least 16 in the lysosomal membranes; the migration distances which correspond to these 16 bands are indicated on the L and PM tracings by vertical bars. The apparent molecular weights of the 16 bands are presented in Table 3.

Characterization of lysosomal membrane proteins

Lysosomal membranes contained less than 20% of the lysosomal protein (Table 1) and were enriched in ATPase activity and thiol groups (Table 2). We have previously suggested that the ATPase may function as an ATP-driven proton pump which serves to maintain an acidic milieu inside the lysosome (Schneider & Cornell, 1978). The possibility that membrane thiol groups are necessary for lysosome function or regulation thereof merits consideration. Conceivably, protein degradation might be regulated by the oxidation-reduction status of lysosomes. Lysosomal membranes isolated from fed and starved rats were found, however, to contain similar amounts of thiols.

The proteins of lysosomal membranes were further characterized by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. As Fig. 1 illustrates, a number of polypeptides were found in the membrane fraction, and the distribution of bands from membranes differed distinctly from those of plasma membranes and from whole lysosomes. Major lysosomal membrane species were observed at migration distances corresponding to apparent mol.wts. of 115000, 105000, 90000, 75000 and 60000. The presence of membrane glycoproteins was examined

in gels after periodic acid/Schiff staining (Fig. 2). Five major and about eight minor polypeptides were evident. The major ones corresponded to apparent mol.wts. of 125000, 115000, 90000, 75000 and 60000. The apparent molecular weights of the membrane proteins are presented in Table 3. For simplicity's sake, it is assumed that a given band represents a single protein species: Coomassie Blue-stainable bands and periodic acid/Schiff-stainable bands of similar molecular weight were given the same number.

Topography of membrane proteins in lysosomes

When lysosomes were incubated with [^{125}I]iodide, membranes were significantly labelled only when lactoperoxidase was included in the iodination mixture (Table 4). Inasmuch as the soluble fraction of lysosomes contains detectable peroxidase activity, the dependency of iodination on added lactoperoxidase may indicate that the lysosomal membrane is impermeable to iodide. The iodinated membrane proteins were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and ^{125}I was found in association with most of the protein bands (Fig. 3). This result suggests that many membrane

Fig. 2. Glycoproteins of lysosomal membranes

Membranes were prepared and electrophoresed as in Fig. 1, except that the bands were located with periodic acid/Schiff reagent (Zacharius *et al.*, 1969). The amount of sample was 0.025 mg of protein. The numbered vertical bars represent bands whose molecular weights are presented in Table 3.

Table 3. Apparent molecular weights and relative abundance of lysosomal membrane proteins

Membranes were dissociated in sodium dodecyl sulphate as in Fig. 1. Apparent molecular weights were obtained by comparison of the positions of bands with those of β -galactosidase (130 000 mol.wt.), bovine serum albumin (68 500), ovalbumin (45 000), trypsin (23 500) and haemoglobin (16 000). Protein bands were detected with Coomassie Blue or periodic acid/Schiff stains.

Band	Apparent molecular weight	Abundance (% of total area)	
		Coomassie Blue	Periodic acid/Schiff
1	200 000	1	—
2	190 000	1	—
3	180 000	1	2
4	155 000	2	4
5	135 000	4	4
6	125 000	5	10
7	115 000	9	15
8	105 000	7	—
9	90 000	14	12
10	75 000	9	8
11	65 000	4	5
12	60 000	7	8
13	50 000	4	4
14	45 000	2	3
15	35 000	4	4
16	30 000	2	2
		76	81

Table 4. Radioactivity of lysosomal membranes after iodination of whole lysosomes in the absence and presence of added lactoperoxidase

Lysosomes, isolated as in Table 1, were iodinated in the presence of 0.5 mM-KI and 0.2 mM-H₂O₂ (see the Experimental section). Membranes were prepared, as in Table 2, from lysosomes which were intact after iodination. Radioactivity was measured by scintillation counting in Triton/toluene (Meade & Stiglitz, 1962; Lazarow & de Duve, 1971).

Addition	Radioactivity		
	Total Na ¹²⁵ I added (c.p.m.)	Incorporated in membranes	
		(c.p.m.)	(%)
None	196 × 10 ⁶	2700	0.0014
Lactoperoxidase	235 × 10 ⁶	4.7 × 10 ⁶	2.0

Fig. 3. Effect of [^{125}I] iodination of lysosomes on distribution of labelled membrane proteins in sodium dodecyl sulphate/polyacrylamide gels

Fresh lysosomes were iodinated in the presence of lactoperoxidase (a) and in its absence (b) and then intact lysosomes were reisolated. Membranes were subsequently prepared from the reisolated lysosomes and electrophoresed as described in the Experimental section. The amount of sample was $8\mu\text{g}$ of protein for membranes from lysosomes iodinated by lactoperoxidase and $85\mu\text{g}$ for the control. The gels were sliced in 2mm sections, dissolved in H_2O_2 and counted for radioactivity in Triton/toluene (Lazarow & de Duve, 1971). The vertical bars and numbers correspond to protein bands whose molecular weights are presented in Table 3.

Fig. 4. Effect of cross-linking lysosomes to bovine serum albumin on distribution of membrane proteins in sodium dodecyl sulphate/polyacrylamide gels

Freshly prepared lysosomes were made to react with activated bovine serum albumin and then the lysosomes remaining intact were reisolated as described in the Experimental section. Membranes were prepared and electrophoresed, and in each case [control (a) and cross-linked (b)], $80\mu\text{g}$ of protein was applied to the gel. Protein bands were detected by staining with Coomassie Blue. The vertical bars and numbers correspond to protein bands whose molecular weights are presented in Table 3.

proteins are exposed to the cytoplasm. To substantiate this result, methods of different specificities were used next.

Toluene-2,4-di-isocyanate is a bifunctional reagent (Schick & Singer, 1961) which has been used to activate bovine serum albumin for use as an impermeant cross-linking reagent (Eytan & Schatz, 1975). Lysosomes were incubated with activated bovine serum albumin, lysosomes that remained intact were reisolated, and membranes were prepared. Analysis of the membrane proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that most proteins failed to enter the gel (Fig. 4). We attribute this to extensive cross-linking. Measurement of relative peak areas suggests that protein bands 6, 7, 9 and 10, corresponding to apparent mol.wts. of 125000, 115000, 90000 and 75000 respectively, are

especially reactive with activated bovine serum albumin and are therefore exposed.

Treatment of lysosomes with trypsin has a limited effect on the membrane proteins (Fig. 5). By comparison of relative peak areas we concluded that protein bands 1, 3 and 15, having apparent mol.wts. of 200000, 180000 and 35000 respectively, are exposed. In addition, protein bands 11 and 12, with apparent mol.wts. of 65000 and 60000, are perhaps susceptible to trypsin. Peaks 4 and 8, corresponding to mol.wts. of 155000 and 105000, were diminished when lysosomes were incubated in either the presence or the absence of trypsin. These are presumably digested from the inside by lysosomal cathepsins and are candidates for proteins exposed to the inside.

Finally lysosomes were treated with subtilisin, and extensive digestion of both Coomassie Blue- and periodic acid/Schiff-stainable membrane proteins was seen. Small amounts of bands 6, 7, 9 and 11 were evident.

Fig. 5. Effect on lysosomal membrane proteins of treating lysosomes with trypsin
Lysosomes were incubated at 37°C with trypsin for the indicated times (0–35min). Afterwards membranes were prepared from the lysosomes that remained intact and were electrophoresed. In each case 50 µg of protein was applied to the gel. Protein bands were detected by staining with Coomassie Blue. The molecular weights of the protein bands numbered 1–16 are presented in Table 3.

Table 5. Summary: membrane proteins located at the cytoplasmic surface of lysosomes
Surface localization was determined by iodination in the presence of lactoperoxidase (¹²⁵I), cross-linking with bovine serum albumin (TC-BSA), and susceptibility to trypsin or subtilisin. The number of pluses denotes the relative degree of exposure.

Band	Surface localization			
	¹²⁵ I	TC-BSA	Trypsin	Subtilisin
1	+++		+++	+++
2	+			+++
3	+		+++	+++
4	+++	+		+++
5	+++			+++
6	+	+++		++
7	+	+++		++
8	+			+++
9	+++	+++		++
10	+++	+++		+++
11	+++		+	++
12	+	+	+	+++
13	+			+++
14	+++			+++
15	+	+	+++	+++
16	+	+		+++

Discussion

Previous studies of the lysosomal membrane focused on lipid and carbohydrate composition (Henning *et al.*, 1970, 1973; Henning & Stoffel, 1973; Thines-Sempoux, 1973; Milsom & Wynn, 1973). Here we have been concerned with integral membrane proteins. These proteins were found to be in large part exposed and to have a high content of thiol groups, 119 nmol/mg of protein. In comparison, rat liver microsomal fractions are reported to have

36 nmol of thiol groups/mg of protein (Isaacs & Binkley, 1977). This thiol content and the content of ubiquinone in the lysosomal membrane (Henning & Stoffel, 1972) pose interesting possibilities: (1) lysosomal activity may be regulated by oxidation–reduction state, or (2) lysosomal acidity may be maintained by an oxidation–reduction system similar to the protonmotive Q cycle proposed for mitochondria (Mitchell, 1975).

Our observations on topography are summarized

in Table 5 and indicate that most, if not all, of the membrane proteins of rat liver lysosomes are exposed to the cytoplasm. We are concerned with the origin of these membrane proteins. One possibility is that these exposed proteins represent residual material which became associated with the lysosomal membrane as a consequence of fusion with endocytic or autophagic vacuoles and then escaped hydrolysis by virtue of their location. The acid proteinases inside the lysosome would presumably be unable to digest proteins located on the other side of the lysosomal membrane and exposed to the cytoplasm. Although, at present, we cannot rule this possibility out, we suspect that it is not applicable and that the exposed proteins are authentic lysosomal proteins, because (1) these proteins appear as discrete bands on sodium dodecyl sulphate/polyacrylamide gels and are of relatively high molecular weight, (2) proteins spared from hydrolysis appear to return to their original source, and recycle, e.g. the low-density-lipoprotein receptor (Brown & Goldstein, 1978), and (3) the gel pattern of the lysosomal membrane is distinctly different from that of plasma membrane, from which proteins of endocytic vesicles would originate.

The membranous ATPase is included in the class of exposed proteins (Schneider, 1977). Whether the ATPase is transmembranous, as one would expect for an ion pump, is uncertain. However, the autolysis of membrane proteins, which occurs when lysosomes are incubated alone, is evidence that some membrane proteins may be transmembranous, e.g. that with an apparent mol.wt. of 105000.

The exposed location of glycoproteins may be an indication of their role in membrane-membrane recognition and fusion. One might be able to test this possibility further in a fusion system *in vitro*. Fusion experiments have been successful with phagolysosomes from *Acanthamoeba castellanii* (Oates & Touster, 1976). Interestingly, an exposed glycoprotein also occurs in pancreatic zymogen granules (Lewis *et al.*, 1977). Further examination of systems *in vitro* may permit a definition of the role of surface proteins and carbohydrates in intracellular recognition of organelles.

We are grateful for financial support from the National Science Foundation, U.S.A. (grant PCM 77-02635). Discussions with Dr. E. W. Westhead, Dr. G. E. Lienhard, Dr. B. L. Trumpower and Dr. O. A. Scornik were helpful. We appreciate the technical assistance of Mr. E. Cornell and Mr. M. Levens and the typing of Joan Eaton.

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