Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1261–1264, March 1983
Biochemistry

Endogenous inhibitors of lysosomal proteinases

(lysosomal membranes/cathepsin B/cathepsin L/cathepsin M/fructose 1,6-bisphosphatase converting enzyme)

S. PONTREMOLI*, E. MELLONI*, F. SALAMINO*, B. SPARATORE*, M. MICHETTI*, AND B. L. HORECKER†

*Institute of Biological Chemistry, University of Genoa, Genoa, Italy; and †Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Contributed by B. L. Horecker, December 10, 1982

ABSTRACT **Specific inhibitors of three lysosomal proteinases are present in the cytosolic and lysosomal compartments of rabbit liver. The cytosolic inhibitors, purified by chromatography on DEAE-Trisacryl and Sephadex G-75, show specificities toward cathepsin M, cathepsins B and L, and fructose 1,6-bisphosphatase converting enzyme (CE), respectively, and are designated I_M, $I_{B/L}$, and I_{CE}. Inhibitors with similar specificities have been isolated from the intralysosomal compartment. Two of these inhibitors, I_M and I_{CE}, are also present in the lysosomal membranes. The lysosomal distribution parallels that of the respective proteinases. The inhibitors are polypeptides with molecular weights of 5,000–10,000 for the two forms of $I_{B/L}$, 12,500 for I_M, and 10,000–40,000 for the I_{CE} species.**

Endogenous inhibitors of lysosomal proteinases have been identified in a variety of rat and human tissues (1) as well as in rat (1) and human (1–3) serum. (For reviews see refs. 4 and 5.) Intracellular inhibitors of lysosomal proteinases specific for cathepsins B and H were recently identified and partially purified from rat lung and hog kidney by Lenney *et al.* (1). More recently an inhibitor of lysosomal thiol proteinases, most effective against cathepsin H, has been isolated in homogeneous form from rat liver cytosol (6–8). Similar cytosolic inhibitors of thiol proteinases have been partially purified from bovine spleen (9).

We report here the characterization of at least three distinct inhibitors, each specific for a different lysosomal proteinase or set of proteinases, isolated from rabbit liver cytosol and from both the soluble and membrane compartments of rabbit liver lysosomes. One cytosolic inhibitor, which may be identical to that reported by earlier workers, inhibits the activities of cathepsins B, L, and M. Two other inhibitors, one specific for fructose-1,6-bisphosphatase (Fru-P_2ase)-converting enzyme (CE) and the other specific for cathepsin M, were also isolated from the cytosol fraction. Specific inhibitors for cathepsins B and L, for cathepsin M, and for CE were also isolated from the soluble fraction after disruption of lysosomes. The lysosomal membranes contained inhibitors for CE and cathepsin M. The presence of specific lysosomal inhibitors of lysosomal proteinases has not been reported previously, to our knowledge.

MATERIALS AND METHODS

Materials. Fru-P_2ase (10) and aldolase (11) were purified from livers of fed rabbits as previously described. The purified enzymes were stored at 4°C as suspensions in 80% saturated ammonium sulfate. Before use, the protein was collected by centrifugation and dissolved in 10 mM sodium acetate, pH 6.0, and the solution was dialyzed against the same buffer. CE_2 and cathepsins B, L, and M from rabbit liver were purified by chromatography on Ultrogel AcA 34 and CM-cellulose as described (12, 13).

Fru-P_2, NADP, cysteine, trypsin, subtilisin Carlsberg (type VIII), benzoyl-L-arginine β-naphthylamide (BANA), concanavalin A immobilized on agarose, and methyl α-D-mannoside were purchased from Sigma. Sephadex G-75 was obtained from Pharmacia. DEAE-Trisacryl M was from LKB. CM-cellulose (CM 11) and DEAE-cellulose (DE 32) were obtained from Whatman. Triton WR1339 (injectable) was from Serva (Heidelberg). Standard proteins for molecular weight determination were obtained from Boehringer Mannheim. All other chemicals were reagent grade.

Methods. *Enzyme assays.* Aldolase activity was assayed as described by Gracy *et al.* (14). Fru-P_2ase activity was measured spectrophotometrically at pH 9.2 in the presence of 2 mM $MgCl_2$ as described (15). For each enzyme the unit of activity was defined as the amount required to convert 1 μmol of substrate per min under the standard assay conditions.

Assay of lysosomal proteinases. CE activity was assayed at pH 5.0 with Fru-P_2ase as the substrate. The reaction mixture (0.1 ml) contained 50 mM sodium acetate buffer at pH 5.0, 0.1 mg of Fru-P_2ase, 5 mM cysteine, and 5 μg of purified CE. At 0 time and after 30 min, aliquots were removed and assayed for Fru-P_2ase activity at pH 9.2. One unit of CE activity was defined as the amount that would increase the specific activity of Fru-P_2ase by 1 unit/mg in a 1-hr period.

Cathepsin B and L activities were assayed with BANA as substrate. The assay mixtures (0.25 ml) contained 50 mM sodium acetate buffer at pH 5.0, 5 mM cysteine, 2 mM BANA, and 5 μg of cathepsin B or L. The mixture was incubated for 30 min at 37°C and free β-naphthylamine was determined as described (12). One unit of enzyme activity was defined as the amount required to liberate 1 μmol of β-naphthylamine in 30 min under these conditions. Cathepsin M activity was assayed with aldolase as substrate. The assay mixture (0.1 ml) contained 50 mM sodium acetate buffer at pH 5.0, 5 mM cysteine, 0.1 mg of aldolase, and 1 μg of cathepsin M. One unit of activity was defined as the amount required to inactivate 1 nmol of aldolase in 30 min under these conditions.

Assay of inhibitory activity. Inhibitors were assayed under the conditions used for the evaluation of the activity of the various cathepsins but with a solution of the inhibitor replacing the same amount of buffer. The mixtures were incubated for 5 min at room temperature before the substrate was added. One unit of inhibitor activity was defined as the amount required to inhibit 1 unit of enzyme activity under the specified assay conditions.

Preparation of subcellular fractions and fractionation of the heavy particles. The heavy particle and postlysosomal fractions from livers of control and Triton WR1339-treated rabbits and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: BANA, benzoyl-L-arginine β-naphthylamide; Fru-P_2, fructose 1,6-bisphosphate; Fru-P_2ase, fructose 1,6-bisphosphatase; CE, Fru-P_2ase converting enzyme; kDa, kilodaltons.

membranes from the heavy particle fraction were prepared as described (12). The heavy particles were fractionated by centrifugation through a sucrose density gradient (16). Fractions (1.25 ml) were collected through a pinhole in the bottom of the tube and 1 ml of each fraction was dialyzed overnight against distilled water. To assay for the presence of inhibitors, the pH of each fraction was adjusted to 4.0 and the fractions were heated at 90°C for 10 min. The pH was then brought to 6.0 and the precipitated proteins were removed by centrifugation. The clear supernatants were assayed for proteinase inhibitory activity as described above.

Marker enzymes were assayed with aliquots of the unheated fractions as follows: cathepsin C as described (12), catalase by the method of Snyder *et al.* (17), and NADH oxidase by the method of Mackler (18). Protein was determined by the method of Lowry *et al.* (19).

RESULTS

Isolation of Specific Inhibitors from Rabbit Liver Cytosol. The postlysosomal fraction, heated at pH 2.0 and chromatographed on DEAE-Trisacryl, yielded three peaks of inhibitor activity (Fig. 1). These were designated cytosolic I_{CE} (peak 1), cytosolic I_M (peak 2) and cytosolic $I_{B/L}$ (peak 3) on the basis of their specificity for these lysosomal proteinases or classes of proteinases. Pooled fractions from each peak were further purified by chromatography on Sephadex G-75 (Fig. 2). The fractions containing cytosolic I_{CE}, corresponding to a molecular weight of approximately 40,000, showed no inhibitory activity against cathepsins M, B, or L (Fig. 2*A*). The fractions containing cytosolic I_M, corresponding to a molecular weight of approximately 12,500, did not inhibit CE or cathepsins B and L (Fig. 2*B*). The inhibitor designated cytosolic $I_{B/L}$ also emerged as a single peak, corresponding to a molecular weight of 12,500 (Fig. 2*C*). These fractions also inhibited the activity of cathepsin M. Attempts to separate these three activities by hydrophobic chromatography or with CM-cellulose were unsuccessful.

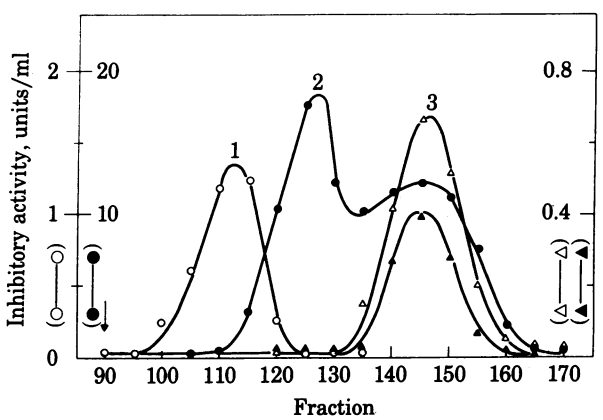

FIG. 1. Separation by ion-exchange chromatography of cathepsin inhibitors in the postlysosomal supernatant fraction. The postlysosomal supernatant fraction corresponding to 45 g of liver was adjusted to pH 2.0 with 6 M HCl and heated at 90°C for 10 min. The mixture was cooled in an ice bath and the pH was adjusted to 6.0. The precipitated proteins were removed by centrifugation and the clear supernatant (180 ml) was concentrated to 10 ml by ultrafiltration on a YM 5 membrane (Amicon) and applied onto a DEAE-Trisacryl column (2 × 10 cm) equilibrated with 20 mM sodium acetate, pH 6.7, containing 0.1 mM EDTA. All the inhibitor activity was retained by the ion-exchanger. The column was washed with the same buffer solution until the absorbance at 280 nm fell below 0.02. The absorbed proteins were eluted with a linear gradient of NaCl begun at fraction 90 (arrow) and reaching 0.16 M at fraction 170. The flow rate was 1 ml/min and 2-ml fractions were collected. Aliquots (50 μl) of each fraction were analyzed for inhibition of CE (○), cathepsin M (●), cathepsin B (▲), and cathepsin L (△).

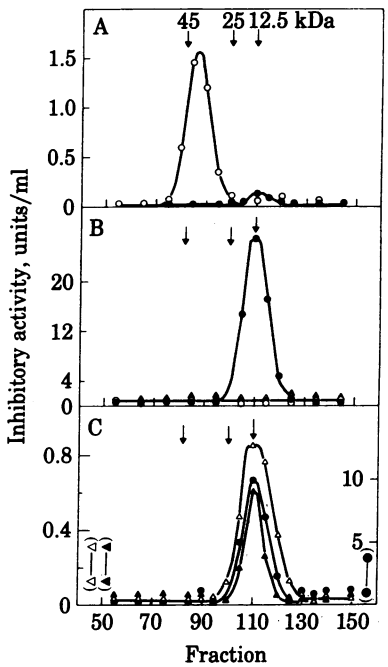

FIG. 2. Gel filtration on Sephadex G-75 of I_{CE}, I_M, and $I_{B/L}$ after separation by DEAE-Trisacryl chromatography. (*A*) Fractions containing cytosolic I_{CE} (fractions 95–115, Fig. 1) were pooled and concentrated to 2 ml by ultrafiltration with a YM 5 membrane and applied to a Sephadex G-75 column (1.6 × 100 cm) equilibrated in 20 mM sodium acetate, pH 6.0, containing 0.1 mM EDTA. The flow rate was 18 ml/hr and 1.4-ml fractions were collected. Aliquots (50 μl) were assayed for inhibition of CE (○), cathepsin M (●), cathepsin B (▲), and cathepsin L (△). No inhibition of the last two was observed in any of these fractions. Fractions 75–100 were pooled and concentrated by ultrafiltration with YM 5 membrane to 2 ml (I_{CE}). The arrows indicate the elution volume of standard proteins: ovalbumin [45 kilodaltons (kDa)], chymotrypsinogen (25 kDa), and cytochrome *c* (12.5 kDa). (*B*) The fractions containing cytosolic I_M (fractions 120–132, Fig. 1) were pooled and concentrated and chromatographed on a Sephadex G-75 column as in *A*. Fractions 100–120 were pooled and concentrated to 2 ml by ultrafiltration with a YM 5 membrane (I_M). (*C*) Fractions 138–160 (Fig. 1) containing cytosolic $I_{B/L}$ were pooled and concentrated and chromatographed on a Sephadex G-75 column as in *A*. Fractions 100–125 were pooled and concentrated to 2 ml by ultrafiltration with a YM 5 membrane ($I_{B/L}$).

Specific Inhibitors Released by Freezing and Thawing of Rabbit Liver Lysosomes. The heavy particle fraction was disrupted by freezing and thawing, centrifuged to remove the membrane fraction, and, after the supernatant solution had been heated at pH 4.0, the soluble proteins were chromatographed on Sephadex G-75 (Fig. 3). Two peaks containing CE inhibitor activity were detected (peaks 1 and 3), corresponding to M_r = 20,000 and M_r = 10,000, respectively. The fractions under peak 1 were collected and designated lysosomal I^a_{CE}. Inhibitor activity for cathepsins B and L also eluted in two peaks (peaks 2 and 4), corresponding to M_r = 12,500 and M_r = 5,000, respectively. The fractions in peak 2 also contained an inhibitor of cathepsin M. The inhibitors of cathepsins B and L in peaks 2 and 4 were designated as lysosomal $I^a_{B/L}$ and lysosomal $I^b_{B/L}$, respectively.

The lysosomal I_M inhibitor in peak 2 was separated from lysosomal $I^a_{B/L}$ by treatment with immobilized concanavalin A, which completely removed lysosomal $I^a_{B/L}$ without loss of lysosomal I_M (Fig. 4). Small amounts of contaminating lysosomal I_{CE}, present in the peak 2 fractions, also failed to bind to the immobilized concanavalin A. The results indicate that lyso-

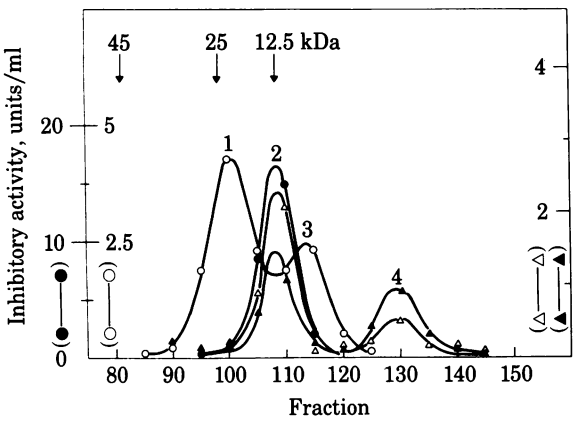

FIG. 3. Separation by gel chromatography of the cathepsin inhibitors present in the rabbit liver intralysosomal compartment. The heavy particle fraction (15 ml), derived from 100 g of rabbit liver, was disrupted by freezing and thawing and the membranes were separated by centrifugation at 100,000 × *g* for 45 min (see *Methods* and ref. 12). The supernatant solution was adjusted to pH 4.0 and heated at 90°C for 10 min. The pH was then brought to 6.0 and the precipitate was removed by centrifugation. The resulting clear supernatant (12.5 ml) was concentrated by ultrafiltration with a YM 5 membrane and chromatographed as described for Fig. 2*A*. Aliquots (5 μl) were assayed for inhibition of CE (○), cathepsin M (●), cathepsin B (▲), and cathepsin L (△). Fractions under each peak were pooled and concentrated to 2 ml by ultrafiltration with a YM 5 membrane (peak 1, fractions 90–101; peak 2, fractions 104–112; peak 4, fractions 125–135).

somes contain four distinct inhibitors, two specific for cathepsins B and L and two others specific for CE and cathepsin M, respectively.

Inhibitors Associated with the Lysosomal Membranes. Two specific inhibitors of lysosomal proteinases were recovered

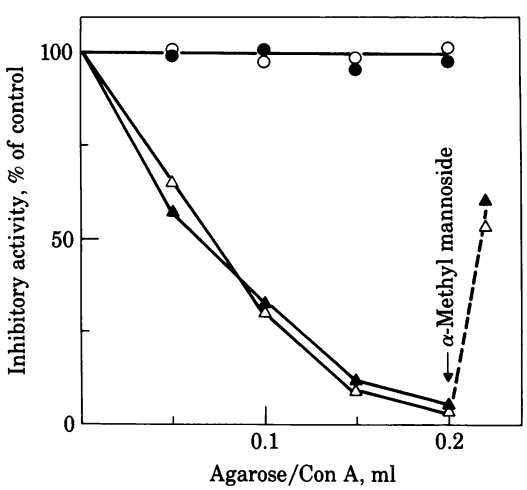

FIG. 4. Separation of lysosomal I_M and $I_{B/L}$ by treatment with immobilized concanavalin A. An aliquot (0.125 ml) of the concentrated lysosomal fraction that inhibited cathepsins B, L, and M (peak 2, Fig. 3) was added to 0.375 ml of a solution containing 20 mM Tris·HCl at pH 6.5, 0.5 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ and the indicated amounts of concanavalin A/agarose. The suspensions were rotated end-over-end for 2 hr at room temperature and the gel was then removed by centrifugation at 600 × *g* for 5 min. The inhibitor activities were assayed by using 10 μl of the clear supernatant solution. ○, CE; ●, cathepsin M; △, cathepsin L; ▲, cathepsin B. The gel was washed three times with 1-ml portions of the same buffer solution and 0.25 ml of 0.5 M methyl α-D-mannoside was added to elute the adsorbed material. The ordinate shows the percent of the original inhibitor activity remaining in the supernatant solution after treatment with immobilized concanavalin A or α-methyl mannoside.

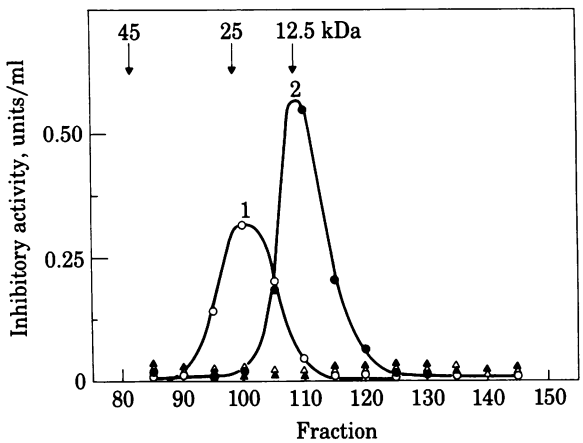

FIG. 5. Separation by gel chromatography of the inhibitors present in the rabbit liver lysosomal membrane fraction. Washed lysosomal membranes, derived from 100 g of liver (122 mg of protein) (see *Methods* and ref. 12) were solubilized by the addition of 1 M NaCl. The membrane extract (12 ml) was adjusted to pH 2 and heated at 90°C for 10 min. The pH was then brought to 6.0 and the precipitated proteins were removed by centrifugation. The resulting supernatant solution (10.5 ml, 6 mg of protein) was concentrated to 1.5 ml by ultrafiltration with a YM 5 membrane and chromatographed on a Sephadex G-75 column as described in the legend to Fig. 2*A*. Aliquots (50 μl) of each fraction were assayed for inhibition of CE (○), cathepsin M (●), cathepsin B (▲), and cathepsin L (△). Fractions 90–102 and 109–120 were separately pooled and concentrated to 2 ml by ultrafiltration with a YM 5 membrane.

from the lysosomal membrane fraction solubilized in 1 M NaCl as described (12). The released proteins, after heating at pH 2, were chromatographed on Sephadex G-75 (Fig. 5). Two peaks of inhibitory activity were recovered; one, corresponding to M_r = 20,000, contained an inhibitor specific for CE (membrane I_{CE}). The second peak, corresponding to M_r = 12,500, contained an inhibitor specific for cathepsin M (membrane I_M). No inhibitor of cathepsin B or cathepsin L was detected in association with the lysosomal membranes.

Molecular Sizes and Properties of Endogenous Inhibitors. The specific inhibitors for cathepsin M, whether isolated from the cytosol, intralysosomal contents, or lysosomal membranes, were of similar size, $M_r \approx 12{,}500$. This was also true for the cytosolic and lysosomal inhibitors of cathepsins B and L, except that the soluble fraction released from disrupted lysosomes contained a second inhibitor of smaller size, $M_r \approx 5{,}000$. The inhibitor of CE present in the cytosol fraction was larger, $M_r \approx$ 40,000, but, after incubation for 60 min at room temperature in the presence of 5 mM cysteine, chromatography of the purified I_{CE} on Sephadex G-75 yielded a major component corresponding with M_r = 20,000 and a minor component with M_r = 10,000. Similarly, lysosomal I_{CE}, isolated from the Sephadex G-75 column in the fraction corresponding to M_r = 20,000, was recovered in the M_r = 10,000 fraction after incubation with 5 mM cysteine. Because cysteine was always present in the assays for lysosomal proteinases, it may be concluded that the active forms of I_{CE} are the monomeric species with M_r = 10,000. The sizes of inhibitors $I_{B/L}$ and I_M were not altered by incubation with cysteine.

All of the inhibitors were destroyed by digestion with trypsin at a ratio of 10:1 (wt/wt, data not shown), which establishes their protein or polypeptide nature. In the crude extracts the inhibitors are heat labile at neutral pH, but stable when heated at pH 2–4 as indicated for each inhibitor by the heating conditions employed for their isolation. Their effects were reversed by dilution of the proteinase–inhibitor complexes.

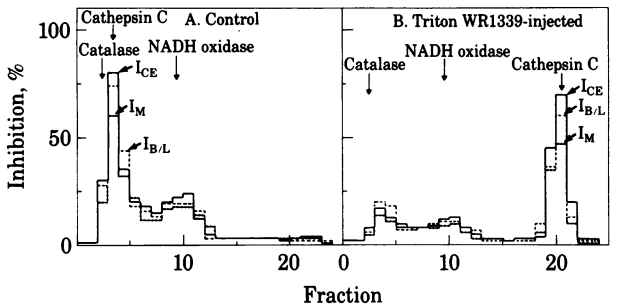

FIG. 6. Sucrose density gradient sedimentation of the heavy particles isolated from control rabbits and from rabbits injected with Triton WR1339. Heavy particles were prepared from livers of control (*A*) and Triton WR1339-treated (*B*) rabbits. A suspension of heavy particles (3 ml, corresponding to 30 g of liver) in 0.25 M sucrose containing 1 mM EDTA was layered on the top of a sucrose density gradient and processed as described (16). The fractions collected were dialyzed overnight against distilled water, the pH was adjusted to 2.0, and the fractions were heated at 90°C for 10 min. The precipitated proteins were removed by centrifugation and the inhibitor activities were assayed with 20-μl aliquots. The locations of peak fraction containing peroxisomes and mitochondria are indicated by the marker enzymes catalase and NADH oxidase, respectively. Assays were also carried out for cathepsin C (16) as the lysosomal marker.

Localization of the Inhibitors in Lysosomes Isolated from Livers of Rabbits Treated with Triton WR1339. In order to confirm the presence of inhibitors in lysosomes, their distribution was analyzed after sucrose density gradient centrifugation of the heavy particle fraction prepared from livers of rabbits injected with Triton WR1339 (Fig. 6). When particles from livers of control rabbits were analyzed by this procedure the inhibitor activity was recovered in two peaks, one of which coincided with the fractions containing cathepsin C, assayed as a lysosomal marker, and another much smaller peak coinciding with the fractions containing NADH oxidase (Fig. 6*A*). In the same experiment carried out with the heavy particle fraction from Triton WR1339-treated rabbits, the inhibitors were recovered mainly in the less-dense Triton-filled lysosome fraction which again coincided with the location of cathepsin C. The same Triton-filled lysosomes were previously shown to contain cathepsins M and CE (16), establishing that the lysosomes contain both the proteinases and the specific proteinase inhibitors.

DISCUSSION

The presence of inhibitors in the cytosol has been described previously (see references cited in the Introduction), with the suggestion that they may serve to protect cytosolic proteins against the action of released lysosomal proteinases or act in other ways to regulate the function of lysosomal proteinases (1).

The finding of inhibitors in the lysosomes was unexpected, particularly because previous workers have reported that these inhibitors are confined to the cytosol (5, 7, 8). Although the inhibitors present in the cytosol and lysosomes are similar in specificity and subunit size after reduction with mercaptoethanol, further experiments are necessary to assess their structural identity.

It may be significant that the distribution of lysosomal inhibitors between the intralysosomal and membrane-bound fractions parallels the distribution of the proteinases on which they act. Thus the intralysosomal space contains inhibitors for CE, cathepsin M, and cathepsins B and L, all of which are present in this compartment. On the other hand, the lysosomal membranes contain inhibitors for CE and cathepsin M, which are present in the membranes, but not for cathepsins B and L, which are not found in the membranes (12, 20). The function of these intralysosomal inhibitors may be to prevent autodigestion of lysosomal enzymes.

The Institute of Biological Chemistry of the University of Genoa acknowledges support from the Italian Consiglio Nazionale delle Ricerche.

1. Lenney, J. F., Tolan, J. R., Sugai, W. J. & Lee, A. G. (1979) *Eur. J. Biochem.* **101**, 153–161.
2. Sasaki, M., Minakata, K., Yamamoto, H., Niwa, M., Kato, T. & Ito, N. (1977) *Biochem. Biophys. Res. Commun.* **76**, 917–924.
3. Järvinen, M. (1979) *FEBS Lett.* **108**, 461–464.
4. Lenney, J. F. (1980) *Curr. Top. Cell. Regul.* **17**, 1–57.
5. Katunuma, N. & Kominami, E. (1980) *Curr. Top. Cell. Regul.* **22**, 71–101.
6. Kominami, E., Wakamatsu, N. & Katunuma, N. (1981) *Biochem. Biophys. Res. Commun.* **99**, 568–575.
7. Hirado, M., Iwata, D., Niinobe, M. & Fujii, S. (1981) *Biochim. Biophys. Acta* **669**, 21–27.
8. Kominami, E., Wakamatsu, N. & Katunuma, N. (1982) *Acta Biol. Med. Ger.* **41**, 69–74.
9. Brzin, J., Kopitar, M., Locnikar, P. & Turk, V. (1982) *FEBS Lett.* **138**, 193–197.
10. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B. & Horecker, B. L. (1978) *Arch. Biochem. Biophys.* **188**, 90–97.
11. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M. & Horecker, B. L. (1979) *Arch. Biochem. Biophys.* **197**, 356–363.
12. Melloni, E., Pontremoli, S., Salamino, F., Sparatore, B., Michetti, M. & Horecker, B. L. (1981) *Arch. Biochem. Biophys.* **208**, 175–183.
13. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M. & Horecker, B. L. (1982) *Arch. Biochem. Biophys.* **214**, 376–385.
14. Gracy, R. W., Lacko, A. G. & Horecker, B. L. (1969) *J. Biol. Chem.* **244**, 3913–3919.
15. Traniello, S., Melloni, E., Pontremoli, S., Sia, C. L. & Horecker, B. L. (1972) *Arch. Biochem. Biophys.* **149**, 222–231.
16. Pontremoli, S., Melloni, E., Michetti, M., Salamino, F., Sparatore, B. & Horecker, B. L. (1982) *Biochem. Biophys. Res. Commun.* **106**, 903–909.
17. Snyder, L. M., Lin, S. C., Palek, J., Bulat, P., Edelstein, L., Srivastava, S. K. & Fortier, N. L. (1977) *Biochim. Biophys. Acta* **470**, 290–302.
18. Mackler, B. (1967) *Methods Enzymol.* **10**, 261–263.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
20. Pontremoli, S., Melloni, E. & Horecker, B. L., in *Lysosomes in Biology and Pathology*, eds. Dean, J., Dingle, J. & Szer, W. (Butterworth, London), Vol. 7, in press.