A Membranous ATPase Unique to Lysosomes

Donald L. Schneider
Department of Biochemistry
University of Massachusetts
Amherst, Massachusetts 01002

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Summary. Intracellular protein degradation presumably occurs in lysosomes and is known to require energy. ATP effects on proteolysis in lysosomes have been demonstrated in vitro and were attributed to transport of protons (1) or transport of proteins (2). Accordingly a lysosomal ATPase was anticipated and is reported herein. The membranous nature of the ATPase as well as its specific activity would seemingly warrant its consideration as the direct link between energy and protein catabolism. However, intact lysosomes display very little ATPase activity and there is no stimulation by uncoupler. Therefore the existence of an ATP-driven proton pump in lysosomes is improbable.

Whereas the dependence of intracellular protein degradation on energy is well established (3), the mechanism of coupling energy to degradation and the actual pathway of degradation remain unknown. Lysosomes are a probable, though unproven, site of protein hydrolysis inasmuch as they contain several active proteases (4). If the concept of the lysosome as the site of protein degradation is correct, then energy might be required by lysosomes themselves in order to transport protons necessary to maintain the acidic pH known to exist inside lysosomes. Additionally, in order to be degraded proteins would be actively transported into lysosomes. It is proposed that an energy-dependent transport system should possess ATPase activity if ATP is the energy source. As a first step in testing this proposition, the presence of a lysosomal, membrane-bound ATPase (ATP phosphohydrolase, EC no. 3.6.1.3) was considered and is the subject of this report.

Materials. Male rats, 100-150 gm, were obtained from Charles River Laboratories Liver lysosomes were prepared by the flotation gradient technique (5) as modified by Leighton et al. (6) in which the crude mitochondrial fraction (ML) is layered beneath a discontinuous sucrose gradient. Triton WR-1339 was purchased from Ruger Chemical Company. Other materials were also obtained from commercial sources.

Table I. Purification of Lysosomes from Rat Liver by Flotation Gradient Centrifugation.

The crude mitochondrial fraction, containing, in addition to mitochondria, lysosomes and peroxisomes, was layered on the bottom of the gradient. After centrifugation, four successive fractions were collected from the top, as described (6). Glucosaminidase was assayed as previously reported (7). ATPase was measured by incubating aliquots of the gradient fractions in a final volume of 0.2 ml containing 40 µmoles Tris-SO_4, pH 8, 200 µg BSA and 0.4 µmole Ca-ATP. After incubation for 10 minutes at 37°, the reaction was terminated by adding trichloroacetic acid to 8%. Protein was removed ty centrifugation, and a portion of the supernatant was analyzed for phosphate by established methods (8). The protein of gradient fractions was measured as described by Lowry (9) except that 0.4% sodium deoxycholate was used to clarify membranous preparations. BSA was used to construct a standard curve.

Fraction	ATPase (µmole/min/gm liver)	ATPase (µmole/min/mg)	Glucosaminidase (µmole/min/gm liver)	Protein (mg/gm liver)
Total liver (E + N)	27.7	0.13	6.17	205.9
Crude mitochondria (ML)	10.64	0.20	3.14	54.7
Gradient:				
1	0.03	----	0.02	0.17
2 (Lysosomes)	0.38	0.32	1.98	1.19
3	0.10	----	0.12	0.28
4	8.95	0.24	0.97	36.5

Results and Discussion. Lysosome preparations isolated by the flotation method from rats treated with Triton WR-1339 are 80% pure and 50 X enriched in marker enzyme activity (6). This degree of purification was confirmed by measurements which demonstrate that the lysosomes were 37to55X enriched in glucosaminidase (Table I). The lysosomes contain, on a specific activity basis, substantial amounts of ATPase activity, shown in Table I. It should be pointed out that ATPase measurements are at neutral pH, and, therefore are not due to the action of non-specific, soluble acid phosphatases. The ATPase activity is absent in fraction 2 when gradients are prepared from rats not treated with Triton WR-1339 (data not shown). Lysosomes are the only organelle affected by Triton (10), and thus the ATPase can be attributed to lysosomes themselves.

Nevertheless, since lysosomes are but a small fraction of liver protein (1.6%) and since the distribution of most ATPase activity is not affected by Triton WR-1339, the possibility that an ATPase adsorbs non-specifically on lysosomes must be considered. A prime candidate would be mitochondrial ATPase because mitochondria are the major constituent of the ML fraction which is applied to the gradient. Therefore, the ATPase activity of lysosomes was tested with oligomycin, a known inhibitor of the mitochondrial enzyme, and found to be relatively insensitive, especially when compared to the mitochondrial ATPase (Table II).

The ATPase activity of lysosomes was previously attributed to plasma membrane $(Mg^{2+} + K^{+})$-ATPase taken up by lysosomes in the process of endocytosis (11). The (Ca^{2+})-ATPase activity of lysosomal membranes reported here is 20 X greater than the previously reported $(Mg^{2+} + K^{+})$-ATPase activity. The difference cannot be accounted for and is not, for example, due to metal ion chelation by 20 mM tartrate in the Kaulen assay. Since lysosomes have ATPase of high specific activity which is insensitive to oligomycin and since the distribution of ATPase activity in sucrose gradients is affected by Triton WR-1339, the activity must represent a bona fide lysosomal enzyme.

The lysosome concept suggests that materials are degraded only after entry

Table II. Effect of Oligomycin on Lysosomal and Mitochondrial ATPases.

Lysosomes were prepared and analyzed as described in Table I. The mitochondria consisted of the "ML" fraction (see 6) and were not free of lysosomes and peroxisomes. A stock solution of 100 µg oligomycin/ml 95% ethanol was prepared and aliquots were added to the assays. ATPase activity was measured as in Table I.

Sample	Oligomycin (µg/ml)	ATPase (µmole/min/mg)
Lysosomes	0	0.177
Lysosomes	1.5	0.146
Lysosomes	5.0	0.104
Mitochondria	0	0.149
Mitochondria	1.5	0.021
Mitochondria	5.0	0.012

Table III. Subfractionation of Rat Liver Lysosomes.

Lysosomes were prepared and analyzed as in Table I and stored at -20°. Lysosomes were thawed and diluted with an equal volume of 0.2 M NaCl, 50 mM Tris-SO_4, pH 8, and 1 mM EDTA. The mixture was then centrifuged in the cold in a 50 Ti Spinco rotor for 40 min. at 35K rpm. The pellets were resuspended in 0.2 M NaCl, 50 mM Tris SO_4, pH 8, and 1 mM EDTA by homogenization with a hand-operated Potter. After a second, identical centrifugation, the final pellets were resuspended in the same manner.

Sample	ATPase (µmole/min)	ATPase (µmole/min/mg)	Protein (mg)
Lysosomes	2.94	0.37	7.90
Sup 1	0.87	0.19	4.66
2	0.23	0.12	1.99
Ppt 2 (membranes)	1.88	1.28	1.47

into the acidic, lysosomal compartment (4). This concept offers at least two means of coupling cellular energy to intracellular protein degradation: maintenance of the lysosomal membrane and transport of protons or proteins across the membrane into the acidic interior. The transport of both protons and proteins has received experimental support (1, 2). A transport system is likely to contain membranous components, and thus it is consistent with the transport concept that most of the ATPase activity fractionated with lysosomal membrane fragments (Table III). The specific ATPase activity of the fragments is greater than 1 µmole/min/mg, a value higher than that obtained with mito-

Table IV. ATPase Activity of Intact Lysosomes and Effects of Uncoupler.

Lysosomes were prepared as in Table I and measurements were carried out in the same day. Sonication was with a Branson sonifier model S 125 equipped with a microtip at power setting 4; 2 ml of sample was sonicated at 0° for 15 sec. In the first experiment ATPase was measured as in Table I. Acid phosphatase was measured with 0.1 M β-glycerol phosphate, pH 5, with 200 µg BSA and sample in a final volume of 0.2 ml. Assay incubation, termination and development were the same as for ATPase. However in the second experiment both enzymes were assayed at pH 6.0, buffered with 0.1 M MES. The uncoupler, 2,4-dinitrophenol (DNP), was added from a 0.1 M stock solution in 95% ethanol.

	Sample	Additions	ATPase (µmole/min/mg)	Acid phosphatase (µmole/min/mg)
Exp. 1	Sonicated lysosomes	None	0.20	0.92
	Intact lysosomes	0.25 M sucrose	0.08	0.12
	Intact lysosomes	0.25 M sucrose plus 0.25 mM DNP	0.11	----
Exp. 2	Sonicated lysosomes	None	0.42	0.46
	Intact lysosomes	0.25 M sucrose	0.13	0.13
	Intact lysosomes	0.25 M sucrose plus 0.25 mM DNP	0.15	0.13

chondria and in agreement with the ATPase being a genuine and major constituent of the lysosomal membrane. The membrane-bound lysosomal ATPase of this report may represent the direct link between energy and degradation. However, results reported below are taken as strong evidence against an ATP-driven proton pump.

It has been postulated that lysosomes use external ATP to drive a proton pump (1). In that case, the ATPase activity of intact lysosomes would be as great as that of ruptured lysosomes or, if not, uncouplers which are known to transport protons across membranes (12) should stimulate the ATPase activity of intact lysosomes. Surprisingly, intact lysosomes have considerably less ATPase activity than lysosomes disrupted by sonication (Table IV). Furthermore the ATPase activity of intact lysosomes was not stimulated to any significant extent by the uncoupler dinitrophenol.

Reijngoud and Tager reported that lysosomes prepared by the Triton WR-1339 method were quite permeable to protons (13). Therefore the diminished ATPase activity of intact lysosomes is all the more surprising. This activity combined with a lack of stimulation by uncoupler means that the existence of an ATP-driven proton pump in lysosomes is improbable. Also, recent data by Gruber et al. (14) cast doubt on the existence of an ATP-driven protein transport system in lysosomes. Therefore the necessity of chemical energy in the form of ATP for lysosomal function must be reconsidered. A remaining possibility worth pursuing is that maintenance of the lysosomal membrane requires energy in which case the ATPase might function in activation of fatty acids.

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